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PROGNOSTIC BIOMARKERS IN GASTRIC CANCER

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ACADEMIC DISSERTATION

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TABLE OF CONTENTS

Table of Contents 5

1 Original publications 9

2 Abstract 11

3 Tiivistelmä (Finnish abstract) 13

4 Abbreviations 15

5 Introduction 17

6 Review of the literature 19

6.1 Epidemiology and incidence 19

6.2 Etiology and risk factors 21

6.2.1 Helicobacter pylori 21

6.2.2 Epstein-Barr virus 21

6.2.3 Hereditary syndromes 21

6.2.4 Dietary and lifestyle factors 22

6.2.5 Earlier gastric surgery 22

6.3 Pathogenesis 23

6.4 Classifications 24

6.5 Clinical manifestations and diagnosis 26

6.5.1 Symptoms 26

6.5.2 Endoscopy 26

6.5.3 Preoperative staging 26

6.6 Treatment 27

6.6.1 Surgery 27

6.6.2 Oncological treatment 32

6.6.3 Early gastric cancer (EGC) and endoscopic techniques 33

6.7 Prognostic factors 34
6.7.1 TNM classification 34
6.7.2 Tumor location and histology 36
6.7.3 Biomarkers 36
6.8 Biomarkers in this thesis 36
6.8.1 PODXL 36
6.8.2 PROX1 37
6.8.3 UCHL5 38
6.8.4 MMP-8 38
6.8.5 TIMP-1 39
7 Aims of the study 41
8 Patients and methods 43
8.1 Patients (I-IV) 43
8.2 Tumor tissue specimens (I-IV) 44
8.3 Immunohistochemistry (I-IV) 45
8.4 Scoring of samples (I-IV) 45
8.5 Serum samples (IV) 46
8.6 Statistical analysis (I-IV) 46
9 Results 47
9.1 Immunohistochemistry (I-IV) 47
9.2 Association with clinicopathological characteristics (I-IV) 48
9.3 Serum MMP-8 and TIMP-1 results (IV) 49
9.4 Survival analysis (I-IV) 49
9.5 Multivariable survival analysis (I-IV) 54
10 Discussion 57
10.1 Biomarkers 57

10.1.1 PODXL 57
10.1.2 PROX1 58
10.1.3 UCHL5 59
10.1.4 MMP-8 and TIMP-1 60
10.2 Strengths and limitations of study materials and methods 62
10.3 Future prospects 63
11 Conclusions 65
12 Acknowledgements 67
13 References 69
14 Original publications 93

1 ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV). These original publications are reprinted here with the kind permission of their copyright holders.

I Laitinen A, Böckelman C, Hagström J, Kokkola A, Fermér C, Nilsson O, Haglund C: Podocalyxin as a prognostic marker in gastric cancer. PLoS One 2015; 10: e0145079.

II Laitinen A, Böckelman C, Hagström J, Kokkola A, Kallio P, Haglund C: High PROX1 expression in gastric cancer predicts better survival. PLoS One 2017; 12: e0183868.

III Arpalahti L[*], Laitinen A[*], Hagström J, Mustonen H, Kokkola A, Böckelman C[**], Haglund C[**], Holmberg CI[**]: Positive cytoplasmic UCHL5 tumor expression in gastric cancer is linked to improved prognosis. PLoS One 2018; 13: e0193125.

IV Laitinen A, Hagström J, Mustonen H, Kokkola A, Tervahartiala T, Sorsa T, Böckelman C[**], Haglund C[**]: Serum MMP-8 and TIMP-1 as prognostic biomarkers in gastric cancer. Tumor Biology 2018; 40: 1010428318799266.

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Publication III is included in the doctoral thesis of PhD Leena Arpalahti (University of Helsinki, 2018).

2 ABSTRACT

Background and aims
Gastric cancer is a highly malignant disease and one of the leading causes of cancer-related mortality worldwide. The course of the disease can vary, making the accurate prediction of its progression difficult. New biomarkers could help us assess cancer aggressiveness and behavior, which would be of value when evaluating the prognosis of each individual patient with gastric cancer. Podocalyxin-like protein (PODXL) is a cell-adhesion glycoprotein associated with an aggressive tumor phenotype and a poor prognosis in several forms of cancer. Prospero homeobox protein 1 (PROX1) is a transcription factor involved in the development of various organs, and also plays an important role in colorectal cancer progression. Ubiquitin carboxyl-terminal hydrolase L5 (UCHL5) is a cysteine protease being a part of the protein homeostasis network, and is found both in healthy and in cancer tissue. Matrix metalloproteinase-8 (MMP-8) belongs to the collagenase subgroup of MMPs and is capable of degrading the extracellular matrix (ECM). MMP-8 participates in the proteolytic processing of inflammatory mediators in a wide variety of biological processes and is also associated with various diseases including cancer. Tissue inhibitor of metalloproteinase-1 (TIMP-1) is an important regulator of MMPs and the extracellular environment. The aim of this study was to evaluate the expression and prognostic value of these potential biomarkers in gastric cancer.

Materials and methods
A total of 650 gastric cancer patients underwent surgery at the Department of Surgery, Helsinki University Hospital, between 1983 and 2009 were included in this study. Survival data and death-causes came from patient records, the Population Register Centre of Finland, and Statistics Finland. Two separate tissue microarray (TMA) series prepared from tumor tissue specimens from these patients were the material for immunohistochemical staining of studied antibodies. PODXL immunostaining was studied in TMA series of 337 samples. TMA series of 313 samples were utilized in immunohistochemistry of PROX1 and MMP-8. UCHL5 staining was studied in TMA series of 650 samples. The expression of these markers were evaluated and compared to clinicopathological variables and patient survival. From preoperative blood samples from 233 patients, serum levels of MMP-8 underwent determination with an immunofluorometric assay (IFMA) and TIMP-1 with enzyme-linked immunosorbent assay (ELISA).

Results
PODXL positivity indicated impaired gastric cancer-specific 5-year survival compared to that of patients with PODXL negativity. The result in multivariable analysis remained significant. Patients with high PROX1 expression had significantly better cancer-specific 5-year survival than did those with low expression, a result that remained significant in multivariable analysis. Patients with

positive cytoplasmic UCHL5 tumor expression showed increased survival in the subgroups of small (<5 cm) tumors, of disease stages I-II, and of age over 66. Patients with low (<31 ng/ml) or high (>131 ng/ml) serum MMP-8 level had an unfavorable prognosis compared to those with an intermediate (31-131 ng/ml) serum level. Those patients with high (≥170 ng/ml) serum TIMP-1 levels also had a poor prognosis, and the latter remained significant in multivariable analysis. The molar ratio of serum MMP-8 and TIMP-1 levels with low (<0.07) or high (>0.30) molar ratios predicted a worse prognosis. The prognosis remained the same despite of MMP-8 tissue immunoreactivity.

Conclusions

In gastric cancer tissue, positive PODXL expression is an independent marker of poor prognosis, high cytoplasmic PROX1 expression is an independent marker of better prognosis, and positive cytoplasmic UCHL5 is linked to better prognosis in certain subgroups. For prediction of prognosis in gastric cancer, serum MMP-8 and TIMP-1 are promising biomarkers.

3 TIIVISTELMÄ (FINNISH ABSTRACT)

Taustat ja tavoitteet
Mahasyöpä on maailmanlaajuisesti merkittävä syöpäkuolleisuuden aiheuttaja. Taudin kulku vaihtelee sen asteesta riippuen ja ennusteen määrittäminen yksilöllisesti voi olla vaikeaa. Uudet biomarkkerit saattaisivat auttaa syövän potilaskohtaisen vaikeusasteen ja käyttäytymisen ennustamisessa. Podocalyxin-like protein (PODXL) on solukalvon glykoproteiini. Aikaisemmissa tutkimuksissa sen on osoitettu liittyvän aggressiivisiin syöpäkasvaimiin sekä muutamissa syöpätyypeissä myös huonoon ennusteeseen. Prospero homeobox protein 1 (PROX1) säätelee solun perimän kopiointia eli transkriptiota. PROX1-proteiinilla on merkittävä rooli eri elinten kehityksessä, mutta sen on havaittu myös osallistuvan ainakin paksusuolisyövän kehittymiseen. Ubiquitin carboxyl-terminal hydrolase L5 (UCHL5), solun proteiinien hajotusjärjestelmän entsyymi, liittyy solujen proteiinitasapainon ylläpitämiseen poistamalla ubikitiinia proteasomisubstraateista. Terveen kudoksen lisäksi sitä on löydetty myös vaihtelevia määriä syöpäkudoksista. Matrix metalloproteinase-8 (MMP-8), eli soluväliaineen endopeptidaasi, kuuluu soluväliaineen proteiineja hajottaviin entsyymeihin. Soluväliaineen tuhoamisen lisäksi sen on osoitettu säätelevän tulehduksellisia tekijöitä useissa tautiprosesseissa sekä myös syövässä. Tissue inhibitor of metalloproteinase-1 (TIMP-1) estää metalloproteinaasien toimintaa soluväliaineessa. Väitöskirjatutkimuksen tavoitteena oli selvittää näiden uusien biomarkkerien esiintymistä mahasyövässä sekä niiden mahdollista yhteyttä potilaiden ennusteeseen.

Materiaali ja menetelmät
Aineisto käsittää 650 Helsingin seudun yliopistollisessa keskussairaalassa (HYKS) vuosien 1983 ja 2009 välisenä aikana leikattua mahasyöpäpotilasta. Potilaiden elossaolo- ja kuolinsyytiedot on kerätty potilaskertomuksista, Väestörekisterikeskuksesta sekä Tilastokeskuksesta. Kudossirublokkitekniikassa kootaan useita kudosnäytteitä samalle kudosblokille. Potilaiden syöpäkudosnäytteistä valmistettiin kaksi sarjaa, joita hyödynnettin värjäämällä niitä tutkittujen biomarkkerien vasta-aineilla. Vasta-aineiden ilmentymä arvioitiin ja tuloksia verrattiin tiedossa olleisiin kliinispatologisiin muuttujiin sekä potilaiden ennusteeseen. Neljännessä osatyössä määritettiin myös 233 potilaan verinäytteistä MMP-8:n seerumipitoisuus immunofluorometric assay (IFMA)-menetelmällä sekä TIMP-1:n seerumipitoisuus enzyme-linked immunosorbent assay (ELISA)-menetelmällä.

Tulokset
Mahasyöpäkudoksen positiivinen PODXL-värjäytyvyys ennusti potilaideiden heikompaa 5-vuotiselossaoloennustetta verrattuna potilaisiin, joiden PODXL-värjäys jäi negatiiviseksi. Tulos osoittautui merkitseväksi myös monimuuttuja-analyysissä. Kohtalainen tai voimakas PROX1-värjäytyvyys ennusti potilaille merkittävästi

parempaa 5-vuotiselossaoloennustetta verrattuna potilaisiin joiden värjäysvoimakkuus oli heikko tai negatiivinen ja myös tämä tulos osoittautui tilastollisesti merkitseväksi monimuuttuja-analyysissä. Syöpäsolujen sytoplasman positiivinen UCHL5-värjäytyvyys liittyi parempaan ennusteeseen potilailla joilla oli pieni kasvainkoko (<5 cm), I-II asteen syöpä tai jotka olivat yli 65-vuotiaita. Mikäli seerumin MMP-8-pitoisuus oli matala (<31 ng/ml) tai korkea (>131 ng/ml), ennusti se potilaiden huonompaa ennustetta. Potilailla, joilla oli korkea TIMP-1-seerumipitoisuus (≥170 ng/ml), oli myös huonompi ennuste ja tämä tulos osoittautui merkitseväksi myös monimuuttuja-analyysissä. Syöpäkudoksen MMP-8-värjäytyvyydellä ei ollut yhteyttä potilaiden ennusteeseen.

Johtopäätökset

Positiivinen PODXL-värjäytyvyys potilaan syöpäkudoksessa on itsenäinen huonon ennusteen merkki mahasyövässä. Sen sijaan selkeä sytoplasminen PROX1-värjäytyvyys syöpäkudoksessa liittyy potilaan parempaan ennusteeseen. Positiivinen sytoplasman UCHL5-värjäytyvyys liittyy potilaiden parempaan ennusteeseen tietyissä alaryhmissä. Seerumin MMP-8 ja TIMP-1 ovat myös lupaavia ennusteellisia biomarkkereita mahasyövässä.

4 ABBREVIATIONS

AJCC	American Joint Committee on Cancer
CA	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CI	Confidence interval
CIN	Chromosomal instability
CSS	Cancer-specific survival
CT	Computed tomography
DUB	Deubiquitinating enzyme
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGC	Early gastric cancer
EGD	Esophagogastroduodenoscopy
EGJ	Esophagogastric junction
ELISA	Enzyme-linked immunosorbent assay
EMR	Endoscopic mucosal resection
EMT	Epithelial-to-mesenchymal transition
ESD	Endoscopic submucosal dissection
EUS	Endoscopic ultrasound
GS	Genomic stability
HDGC	Hereditary diffuse gastric cancer
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HIPEC	Hyperthermic intraperitoneal chemotherapy
H. pylori	*Helicobacter pylori*
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IFMA	Immunofluorometric assay
IQR	Interquartile range
miRNA	MicroRNA
MMP-8	Matrix metalloproteinase-8
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
PET	Positron emission tomography
PODXL	Podocalyxin-like protein
PROX1	Prospero homeobox protein 1
ROC	Receiver-operating characteristic
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TIMP-1	Tissue inhibitor of metalloproteinase-1
TMA	Tissue microarray
TNM	Tumor, node, metastasis

UCHL5	Ubiquitin carboxyl-terminal hydrolase L5
Uch37	Ubiquitin C-terminal hydrolase 37; UCHL5
UICC	Union for International Cancer Control
WHO	World Health Organization

5 INTRODUCTION

Cancer is a leading cause of death worldwide, and the number of new cancer cases and deaths is estimated to rapidly increase as the populations grow and live longer and at the same time adopt lifestyle behaviors that increase cancer risk. Globally, gastric cancer is the fifth most common cancer and the third leading cause of cancer-related deaths. Its geographic and socioeconomic diversity of incidence is considerable: about 70% of gastric cancer cases occur in developing countries and about half in eastern Asia (Torre 2015). In Finland, gastric cancer is rare nowadays being responsible of about 2% of all cancers and about 4% of cancer-related deaths (Finnish Cancer Registry).

The incidence and mortality rates of gastric cancer have been substantially declining during recent decades. These changes are assumed to be attributable to the declining prevalence of *Helicobacter pylori* infection due to improved sanitation and antibiotics, better availability of fresh food with less reliance on salt-preserved food (Parkin 2006). Decline in tobacco smoking in developed countries may also have contributed to the fall in gastric cancer rates (Ladeiras-Lopes 2008, Bertuccio 2009).

Despite developments in incidence, diagnostics, and therapeutic options in recent decades, the gastric cancer prognosis still remains poor, especially at advanced stages. The basis of curative treatment is radical surgery. The prognosis is highly TNM-stage-specific with 5-year survival of 57–71% for stage I disease, 33-46% for stage II, 9-20% for stage III, and 5-year survival falls to only 4% for stage IV patients (Edge 2010). Regardless of the new treatment options such as surgery combined with perioperative chemotherapy, gastric cancer remains very difficult to control and to cure. Undoubtedly the need is to identify biomarkers that can help to improve the individual patient's prognosis, and thereby improve choice of the best treatment options.

Gastric cancer is not a single disease, it is now clear that it is multifactorial and highly molecularly diverse. Recently, The Cancer Genome Atlas has described a new classification of four molecular subtypes of gastric cancer (Cancer Genome Atlas Research Network 2014). The subtypes are enriched for selected molecular abnormalities, potentially guiding patient stratification and targeting key pathways driving the tumor in each individual patient.

Biomarkers, particularly tumor markers, may be useful in the early detection of tumors, in assessment of the extent of tumor growth or spread, or in identification of tumor recurrence. They are expressed by the tumor itself or by the host in response to the tumor. Tumor markers may be reactive molecules detected from bodily fluids or tissues and ideally should be both sensitive and specific for the detection of cancer, with a methodology sufficient simple and cost-effective; they should identify

tumor recurrence after treatment and help to determine prognosis and an individual treatment plan for each cancer patient.

This thesis consists of studies on a set of novel, promising prognostic biomarkers in gastric cancer. The project includes immunohistochemical tumor tissue studies of podocalyxin-like protein (PODXL), prospero homeobox protein 1 (PROX1), ubiquitin carboxyl-terminal hydrolase L5 (UCHL5), and matrix metalloproteinase-8 (MMP-8), as well as detection of preoperative serum levels of MMP-8 and tissue inhibitor of metalloproteinase-1 (TIMP-1), in association with different clinicopathological variables and cancer-specific survival.

6 REVIEW OF THE LITERATURE

6.1 Epidemiology and incidence

The first documented cases of possible gastric cancer date back to 1600 BC when they were described in the Ebers Papyrus; later reports of Hippocrates included the words “cancer” and “carcinoma” for the very first time, but he believed that cancer was something attacking the human body from outside and penetrating through the skin to the internal organs. Much later, in 1881, Theodor Billroth performed the first successful gastric cancer operation, a subtotal resection with gastroduodenal anastomosis in Vienna (Santoro 2005).

Nowadays gastric cancer is the world’s fifth most common cancer type, with 952 000 new cases (6.8% of the total) and an age-adjusted incidence of 17.4/100 000 in men and 7.5/100 000 in women in 2012 (Ferlay 2014). The incidence has decreased dramatically in recent decades, especially in the Western and more developed world, since the era when it was the most common cancer worldwide, around 1975. Still, incidence rates vary widely across different countries. The highest incidences occur in men in eastern Asia (China, Japan, Korea) with up to 69 cases per 100 000 (Yamaoka 2008). Incidence rates are also high in central and eastern Europe, and in South America. Rates are lowest in North America and most parts of Africa (Torre 2015).

Worldwide, gastric cancer is the third most common cause of cancer-related death, with approximately 723 000 deaths (8.8% of the total) in 2012. Mortality rates are highest in eastern Asia (24/100 000 in men and 9.8/100 000 in women) and lowest in North America (2.8 and 1.5 respectively). Mortality rates are also high in eastern and central Europe, and in central and South America (Torre 2015).

In Finland, according to the Finnish Cancer Registry, the number of new gastric cancer cases has decreased considerably in recent decades. In 2015, new cases numbered 600, and gastric cancer age-adjusted incidence was 6.5/100 000 for men, 3.8/100 000 for women (Figure 1). The age-standardized 5-year survival of gastric cancer was 24% for men, 29% for women. In 2015, Finland had a total of 453 gastric cancer deaths (3.42/100 000).

Gastric cancer incidence increases with age, with the peak occurring at age 60-80. Among those under 30, gastric cancer is very rare (Theuer 1996, Nakamura 1999). The age-adjusted incidence rate is about twice as high among men as among women (Hartgrink 2009, Torre 2015). Nowadays, the overall number of distal tumors is declining at the same time as more proximal tumors are becoming more frequent, possibly linking the etiology of different tumors with their anatomic location. Increased rates of gastroesophageal reflux and overweight may play a role in the

rising incidence of more proximal gastric tumors, although no evidence exists of any causative relationship (Salvon-Harman 1994, Crew 2006).

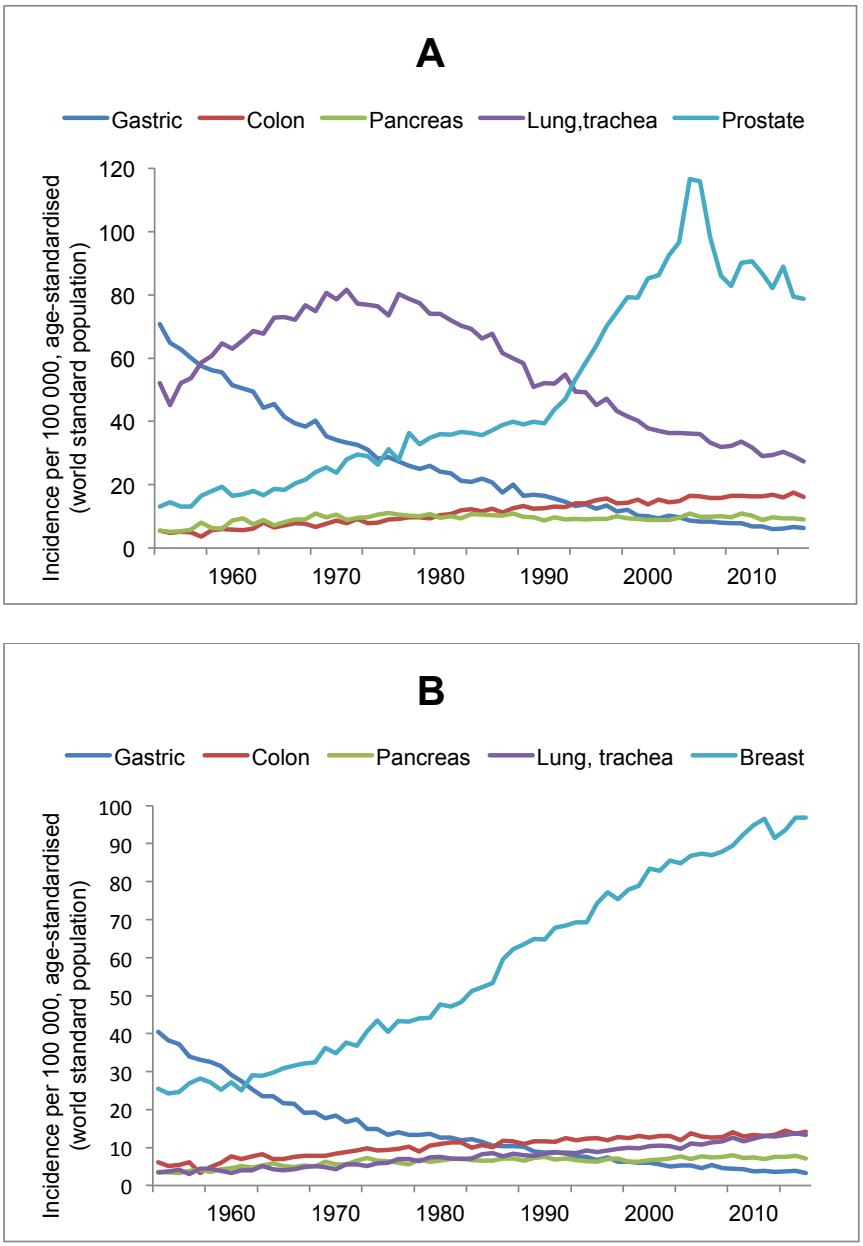


Figure 1. Gastric cancer, pancreatic cancer, and the present (2014) three most common cancer-site age-adjusted incidence in Finland for A) men and B) women. Adapted from the Finnish Cancer Registry, 2015.

6.2 Etiology and risk factors

6.2.1 Helicobacter pylori

Australian scientists Barry Marshall and Robin Warren identified the gram-negative bacterium *Helicobacter pylori* and its presence in a person with chronic gastritis and gastric ulcers, in 1982 (Marshall 1984). In recognition of their discovery, they received the 2005 Nobel Prize in Physiology or Medicine. Strong evidence from various epidemiological and prospective studies has shown that long-term *H. pylori* infection is closely linked to development of atrophic gastritis, which may induce intestinal metaplasia, dysplasia, and gastric cancer (Helicobacter and Cancer Collaborative Group 2001, Uemura 2001, Correa 2007). Atrophic gastritis and intestinal metaplasia then raise the relative risk for development of gastric cancer, ranging from 1.7 in moderate atrophy and 4.9 in severe atrophy, to 6.4 in intestinal metaplasia (Uemura 2001). *H. pylori* infection has been classified by the International Agency for Research on Cancer (IARC) as a type-I carcinogen in gastric cancer (The Eurogast Study Group 1993). The risk for gastric cancer is approximately six-fold higher in populations with 100% *H. pylori* infection than in populations without any infection (Helicobacter and Cancer Collaborative Group 2001). Still, among *H. pylori*-infected individuals only approximately 10% develop gastric ulcer, and only 1-3% gastric cancer (Wang 2014). In addition, differences in *H. pylori cagA* and *vacA* genotypes may explain geographical variations: why some populations have high rates of *H. pylori* infection but low incidences of gastric cancer, such as Africa and South Asia (Yamaoka 2008).

6.2.2 Epstein-Barr virus

The other microbe associated with gastric cancer, Epstein-Barr virus (EBV), is one of the most common viruses in humans and best known as the cause of mononucleosis. It is present in gastric-cancer tumor cells at a rate of approximately 9% of gastric cancers (Murphy 2009). Patients with EBV-positive cancer show a better outcome than do those with EBV-negative tumors (Camargo 2014). The mechanisms underlying this association are unclear, with several theories trying to explain it. A potential immunological basis could exist, in which cytotoxic CD8 lymphocytes may promote eradication of EBV-positive malignant cells (Saiki 1996). An alternative hypothesis is that genetic alterations potentially associated with better survival may be more common in EBV-positive tumors (Wang 2011).

6.2.3 Hereditary syndromes

About 10% of gastric cancers exhibit familial clustering, but only a small number, only 1% to 3%, result from inherited syndromes (Oliveira 2004, Lynch 2005). Hereditary diffuse gastric cancer (HDGC) is a rare and autosomal-dominant inherited form of gastric cancer which typically develops at a young age (Kaurah

2007). HDGC is characterized by a highly invasive diffuse-type tumor, delayed presentation, and poor prognosis. HDGC represents a prominent molecular abnormality with defective intercellular adhesions which may be the result of loss of expression of the cell adhesion protein E-cadherin (Guilford 1998, Richards 1999, Oliveira 2009). Approximately one-quarter of families with HDGC have an inactivating E-cadherin gene (*CDH1*) germline mutations. Estimated lifetime gastric cancer risk in *CDH1* carriers is in men, 67%, in women, 83%. The guidelines recommend *CDH1* testing for 1) families with two or more patients with gastric cancer at any age with one confirmed diffuse cancer type, 2) individuals with diffuse gastric cancer before the age of 40, and 3) families with both diffuse gastric cancer and lobular breast cancer (one diagnosis before the age of 50) (van der Post 2015). Other hereditary syndromes linked to gastric cancer are familial adenomatous polyposis, Lynch syndrome, juvenile polyposis syndrome, Peutz-Jeghers syndrome, Li-Fraumeni syndrome, and gastric hyperplastic polyposis (Varley 1995, Vasen 1996, Keller 1998, Shinmura 2005, Gylling 2007).

6.2.4 Dietary and lifestyle factors

Populations with diets rich in salted and smoked food containing nitrates and nitrites, rich in starch, and with no fresh fruits and vegetables, are at higher risk for gastric cancer. A diet like this may have an effect on acid-catalyzed nitrosation in the stomach and thus cause mechanical damage to the gastric mucosa (Ramón 1993, Tsugane 2007, Krejs 2010, Berretta 2012). In addition, refrigerator use, fruit intake, and gastric cancer mortality have a negative association (Bae 2008, Park 2011).

Smoking is also a reported risk factor for gastric cancer. In a meta-analysis covering 42 articles, current smokers had a relative risk of 1.53 developing gastric cancer comparing to never-smokers (González 2003, Ladeiras-Lopes 2008). The association of alcohol consumption and gastric cancer has been investigated in numerous studies with inconsistent results. Some evidence exists that heavy alcohol drinking may associate with a modestly increased risk for gastric cancer (Duell 2011, Tramacere 2012). Acetaldehyde is the first metabolite of ethanol oxidation and also the most carcinogenic compound of tobacco. It is classified as a carcinogen in humans (Secretan 2009). Aldehydedehydrogenase (ALDH2) and alcoholdehydrogenase (ADH) gene polymorphisms associating with alcohol drinkers enhanced acetaldehyde exposure cause increased cancer risk for gastric cancer (Salaspuro 2011).

6.2.5 Earlier gastric surgery

Gastric cancer risk increases in the gastric stump after earlier distal gastrectomy, even though the reason for surgery has been benign, such as in peptic ulcer disease. The incidence of gastric-stump cancer is estimated at 1-2%, but no prognostic

differences have emerged between stump and primary gastric cancer (Stalnikowicz 1990, Takeno 2014, Thorban 2000).

6.3 Pathogenesis

Intestinal-type gastric cancer develops through a sequence of precursor lesions: chronic gastritis, mucosal atrophy, intestinal metaplasia, dysplasia, and intestinal cancer (Correa 1992). These changes are induced by *H. pylori* infection (Forman 1991, Parsonnet 1991). Five years after diagnosis, the annual incidence of gastric cancer is 0.1% for atrophic gastritis, 0.25% for intestinal metaplasia, 0.6% for mild-to-moderate dysplasia, and 6% for severe dysplasia (de Vries 2008).

Precursors of gastric cancer are gastric adenomas with dysplastic epithelial cells. They can be solitary and occur anywhere in the stomach, but are commonly located in the antrum. Histologically, adenomas are classified into tubular, villous, and tubulovillous types, and they may arise after a history of atrophic gastritis and intestinal metaplasia typically associated with *H. pylori* infection. The risk for cancer development in adenomatous polyps also increases with age and with lesion size. Gastric adenomas occur with similar frequency in men and women (Cristallini 1992, Goddard 2010, Shaib 2013).

Gastric cancer arises as the result of accumulated genomic damage affecting cellular functions vital for cancer development. These hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan 2000, 2011). These genomic changes may arise from two different genomic instability pathways: microsatellite instability or chromosomal instability (Lengauer 1998).

About 15% of gastric cancers are associated with a defect in the mismatch repair system manifested as tumor microsatellite instability (MSI) (Bacani 2005). Throughout cell replication, this repair system notices base pair mismatches, which occur by addition or deletion of a base. Mismatch repair proteins excise the mismatched lesion and resynthesize the DNA before the cell cycle is ready. Silencing of mismatch repair proteins is the most frequent cause of microsatellite instability in sporadic gastric cancer, leading to increased mutation rate at the nucleotide stage (Fleisher 1999). This microsatellite instability has been associated with intestinal-type cancer, tumor location in the antrum, less frequent lymph node metastases, and better survival (Wu 2000, Beghelli 2006).

Roughly 85% of sporadic gastric cancers show chromosomal instability. This manifests in numerical or structural changes of large parts of, or even whole chromosomes, with an aneuploidy DNA pattern. The underlying mechanism of chromosomal instability is largely unknown. Mitotic chromosomal missegregation

and spindle checkpoint errors have been considered (Aguilera 2008, Hartgrink 2009).

Preceding the development of invasive gastric cancer is a stepwise evolution through a cascade of precancerous lesions. Sequential histopathological changes occur in the gastric mucosa including atrophic gastritis with loss of parietal cell mass, intestinal metaplasia, and dysplasia, all of which eventually leads to cancer. This metaplasia-dysplasia-carcinoma sequence is more relevant for the intestinal-type gastric cancer that develops by a cumulative series of genetic alterations similar to those in colorectal cancer (Correa 1992, 2012).

6.4 Classifications

Anatomically, the stomach is divided into several subsites: cardia, fundus, corpus, antrum, and pylorus. Cancers with a midpoint in the stomach situated more than 5 cm distal to the esophagogastric junction (EGJ), or those within 5 cm from the EGJ but not extending into the EGJ or esophagus, are classified as gastric cancers (Sobin 2009). The proximally situated cancers with a midpoint in the esophagus, EGJ, or cardia that extends into the EGJ or esophagus are classified as esophageal cancers. Anatomically, the medial and lateral curvatures are called the lesser and greater curvatures (Edge 2010). Histologically, the wall of the stomach has five layers: the mucosa, submucosa, muscularis propria, subserosa, and serosa. Approximately 95% of gastric tumors are epithelial in origin and classified as adenocarcinomas. Adenosquamous, squamous, and undifferentiated carcinomas are rare (Sarbia 2004).

The Laurén classification stratifies gastric adenocarcinoma into two major histologic types: intestinal and diffuse, describing tumors on the basis of their microscopic configuration and growth pattern (Laurén 1965). Intestinal carcinoma form glands that range from well to poorly differentiated tumors and which grow in expanding patterns and typically arise from chronic atrophic gastritis and intestinal metaplasia (Dicken 2005). Diffuse carcinoma consists of noncohesive tumor cells diffusely infiltrating the gastric wall with little or no gland formation. These cells are usually round and small and may look like signet rings when mucus-containing cells push the nucleus to the cell periphery; they can be arranged as separate single cells or in clusters. Linitis plastica is a morphologic variant of diffuse cancer in which the gastric wall thickens without clear tumor borders. A mixed carcinoma is a tumor that contains approximately equal quantities of intestinal and diffuse components.

These two histological types, intestinal and diffuse, differ in their histologic appearance but also differ in gender ratio, age at diagnosis, and other epidemiologic features (Henson 2004). The diffuse type gastric cancer is more often seen in women and young patients, and is typically situated in the proximal stomach (Laurén 1965). The intestinal type is more common in men and older patients. It tends to arise from precancerous lesions, it is often associated with intestinal metaplasia and *H. pylori*

infection, it is more often situated in the distal portion of the stomach, and it is linked to dietary factors (Kaneko 2001).

The World Health Organization (WHO) classifies gastric adenocarcinoma as tubular, papillary, mucinous, and poorly cohesive, including signet ring cell carcinoma, and uncommon histologic variants (Hamilton 2000). Despite tumors' histological variability, classification is based on the predominant histological pattern that often co-exists with less dominate elements of other histologic patterns.

The Cancer Genome Atlas (TCGA) has suggested a new molecular subtyping of gastric adenocarcinomas into four subtypes based on the presence of Epstein-Barr virus (EBV), microsatellite instability (MSI), genomic stability (GS), and chromosomal instability (CIN) (Cancer Genome Atlas Research Network 2014, Figure 2). These subtypes have distinct genomic features, providing a guide for patient stratification and trials of targeted therapies. This kind of classification offers valuable information about the variability in biological characteristics among gastric cancer but is not applicable for routine clinical diagnostics. Recently, some more straightforward methods may be able to reveal more useful classifications for clinics (Kim 2016, Park 2016, Setia 2016, Ahn 2017, Birkman 2017).

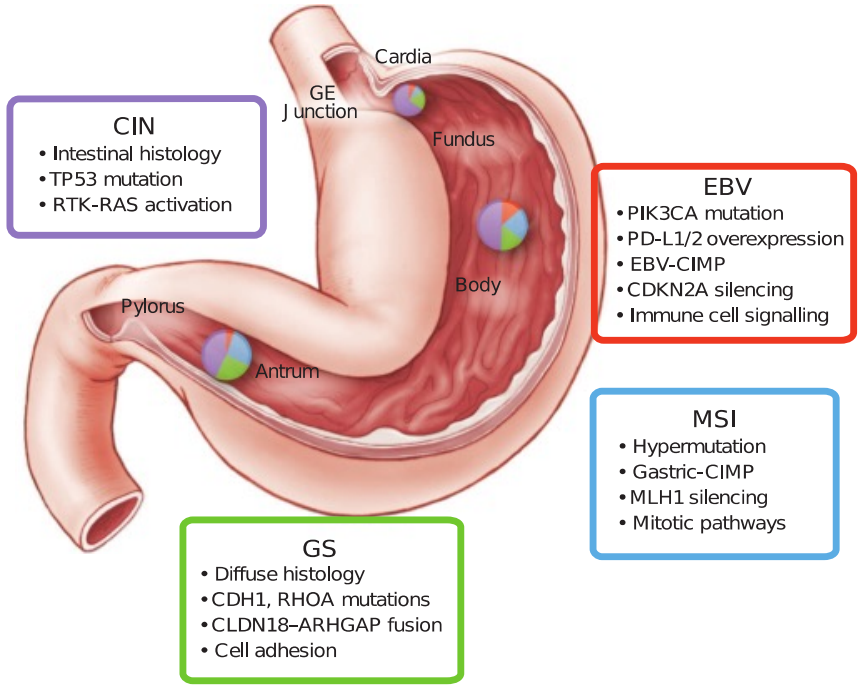


Figure 2. Key features of the new molecular subtyping of gastric adenocarcinoma by TCGA. Reprinted with permission of Springer Nature (Cancer Genome Atlas Research Network 2014).

6.5 Clinical manifestations and diagnosis

6.5.1 Symptoms

As early pathognomic symptoms are lacking, patients often already show advanced gastric cancer at diagnosis. Nonspecific early symptoms may be nausea, mild upper gastrointestinal distress or heartburn, flatulence, excessive belching, and abdominal pain or fullness after meals. Weight loss, vomiting, dysphagia, fatigue, gastrointestinal bleeding, and a palpable abdominal mass are usually signs of advanced cancer (Catalano 2009, Hartgrink 2009). Chronic anemia may correlate with ulcerated lesions. Distal tumors may cause obstructive symptoms, whereas proximal tumors typically manifest with nausea and vomiting (Dicken 2005).

Metastatic manifestations may be liver enlargement, presence of ascites, jaundice, and palpable lymph nodes in the supraclavicular region (Virchow's node), in the left axilla (Irish's node), or in the periumbilical region (Sister Mary Joseph node). Peritoneal spread may cause ovarian metastases (Krukenberg tumor) or a palpable pelvic mass (Blumer's shelf) (Dicken 2005, Catalano 2009). Paraneoplastic syndromes are rare, but include dermatomiositis, acantosis nigricans, microangiopathic hemolytic anemia, and chronic intravascular coagulation leading to arterial and venous thrombi (Trousseau's syndrome) (Catalano 2009).

6.5.2 Endoscopy

Esophagogastroduodenoscopy (EGD) is the method of choice for gastric cancer diagnosis, as it allows direct visualization of tumor appearance, size, location, and the extent of mucosal involvement, and at the same time enables to photography and biopsies from suspected lesions (Dicken 2005).

Endoscopic ultrasound (EUS) can help with tumor staging by providing information about depth of tumor invasion and allowing evaluation of perigastric lymphadenopathy (Willis 2000). EUS seems to be most effective method to differentiate stages T1 to T2 from stages T3 to T4 (Kwee 2007).

6.5.3 Preoperative staging

The presence of possible metastases determines treatment, and computed tomography (CT) is the most frequent modality for gastric cancer staging (Halvorsen 1996, Angelelli 2001). CT can detect liver metastases and regional and distant lymphadenopathy, and can show signs of tumors' direct invasion into adjacent structures. Intravenous contrast aids in identifying solid-organ metastases and lymph-node spread.

For preoperative staging, what is vital to assess is whether the cancer is suitable for radical surgical resection. Even though improved imaging techniques enable staging more adequately than previously, CT alone is insufficiently sensitive to detect or exclude peritoneal metastases. In patients with gastric cancer, the sensitivity of CT to detect metastatic lymph nodes varies from 62.5% to 91.9% (Kwee 2009). Staging laparoscopy is an adjunct to imaging of patients being considered for curative surgery (Leake 2012, Burbidge 2013, Machairas 2017). Staging laparoscopy may change the surgical treatment plan even in 20 to 30% of cases, and may help to reduce perioperative mortality, eliminating nontherapeutic laparotomies (Smith 2007, Coburn 2010, Shelat 2012).

Magnetic resonance imaging (MRI), even though its accuracy in tumor staging is at least similar to that of CT, has limited use in the staging of the primary due to difficulties with motion artifact, cost, required time, and lack of an appropriate oral contrast agent (Sohn 2000, Motohara 2002).

Positron emission tomography (PET), despite its ability to visualize areas of enhanced metabolic activity within tissues, has been assumed to have a low detection rate for diagnosis of primary gastric cancer, especially in its early stage and in gastric-cancer types that are less metabolically active. PET appears, however, to be more specific for detection of metastatic lymph nodes, peritoneal lesions, and bone metastases as compared to CT alone (Gauthé 2015, Malibari 2015, Kawanaka 2016). PET-CT is a modality that combines these two techniques.

6.6 Treatment

Treatment planning is always done individually and must take into account the stage of the disease, co-morbidities, performance status of the patient, and the patients' own wishes and expectations. An algorithm of different treatment options is in Figure 3. Multidisciplinary treatment planning is the recommendation before any treatment decision. The multidisciplinary team should include surgeons, medical and radiation oncologists, radiologists, and pathologists (Smyth 2016).

6.6.1 Surgery

Surgery is the first-line therapy for curing gastric cancer. Subtotal gastrectomy is suitable for distal cancer if a macroscopic proximal margin of 5 cm can be achieved between the tumor and EGJ. For diffuse cancer, a margin of 8 cm is the recommendation. Otherwise, the choice is total gastrectomy. Evidence exists that both procedures, subtotal and total gastrectomy, show similar survival and mortality rates. Subtotal gastrectomy, associated with better nutritional status and quality of life, should be the procedure of choice, provided that the proximal margin of the resection is disease free (Gouzi 1989, Bozzetti 1999).

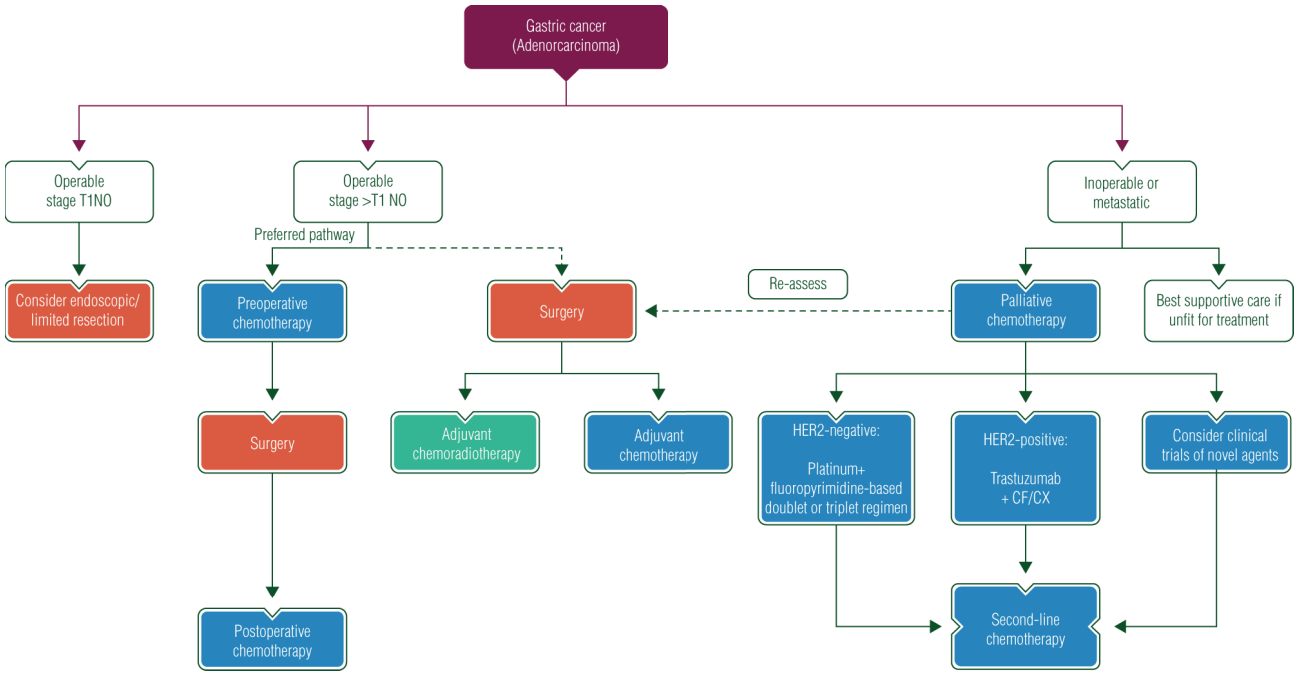


Figure 3. Gastric cancer treatment algorithm. Reprinted with permission of Oxford University Press (Smyth 2016).

The extent of gastric resection has classically been described based on the proximity of the dissected lymph nodes (Figure 4). D0 resection means no nodes are removed, and is typical in the case of palliative resection. In D1 resection, the perigastric nodes along the lesser and greater curvature are removed, together with the omentum. D2 dissection indicates the removal of nodal tissue along the left gastric, common hepatic, celiac, and splenic arteries. For D3 lymphadenectomy, nodes from the porta hepatis, the hepaticoduodenal ligament, and the periaortic and retropancreatic regions must be removed (Hartgrink 2009, Japanese Gastric Cancer Association 2011).

The suitable extent of lymphadenectomy for curative surgery has been the subject of considerable debate over recent decades (Tanizawa 2010). It is conceivable that removal of a large number of lymph nodes improves survival. A limited number of randomized controlled trials from the Western world have focused on this issue. The first results of the prospective randomized Dutch trial comparing D1 with D2 lymphadenectomy, indicated significantly higher mortality after a D2 dissection (10 vs. 4%) (Bonenkamp 1995). At the same time, The Medical Research Council Gastric Cancer trial demonstrated that the number of splenectomies and pancreatectomies, which have been shown to increase postoperative mortality, were also higher in the D2 group than in the D1 group (Cuschieri 1996). Similarly, a recent Italian study failed to demonstrate any survival advantage with D2 dissection, although they suggested a trend towards a benefit from D2 resection in disease-specific survival for patients with T2-T4 tumors with positive lymph nodes (Degiuli 2014). After 11 and 15 years of follow-up, the Dutch study group revealed no significant differences in overall survival. However, when they analyzed cause-specific survival at 15 years, gastric cancer-related death was significantly lower after D2 (37%) than after D1 (48%) dissection ($p=0.01$), suggesting that when postoperative mortality can be avoided, D2 lymphadenectomy improves survival after gastric cancer resection (Hartgrink 2004, Songun 2010). Hereby, D2 dissection for a medically fit patient in experienced, high-volume centers should be the recommended type of surgery in advanced, resectable gastric cancer (Dikken 2011, Smyth 2016). Dissection more extended than D2 seem to have no survival benefit (Sasako 2008).

Since 1991, laparoscopic surgery has been adopted for gastric cancer treatment, starting in Asian countries. In its early years, only early and distal cancers were treated by a laparoscopic method. However, as surgeons gained more experience, more extensive procedures become more common. Laparoscopic surgery seems to be associated with quicker return of gastrointestinal function, faster ambulation, earlier discharge from hospital, and has comparable complications and recurrence rate to those of open surgery. However, the length of operating time for laparoscopy remains longer (Shehzad 2007, Chen 2014). Discussion of adequate lymph node dissection with a laparoscopic approach involves evidence that lymph node dissection for both approaches is comparable (Quan 2016, Chen 2017).

In gastric cancer surgery, avoiding postoperative mortality is a challenge, especially when performed in countries with lower incidence, leading naturally to lower exposure of hospitals and surgeons to resectable gastric cancer cases. Many studies have analyzed the relation between hospital volume and outcome, and found that increased surgeon´s and hospital volumes are associated with lower postoperative mortality and higher survival rates, both in Western countries and in Asia (Begg 1998, Birkmeyer 2002, Dikken 2011,2013). For example, in Denmark, gastric cancer surgery centralization has led to a significant decrease in postoperative mortality and an increase in the number of patients with at least 15 lymph nodes examined (Jensen 2010). Centralization of gastric cancer surgery to five university hospitals is currently implemented also in Finland (Finnish Ministry of Social Affairs and Health, 2017).

In locally advanced or metastatic disease, palliative resection of the primary tumor or its metastases is not recommended in general (Smyth 2016). The primary goal in palliation is relieving symptoms and improving quality of life. Regardless, sometimes surgery is still needed to relieve difficult symptoms such as bleeding or obstruction. Possible procedures for palliative surgery are resection without lymph node dissection, gastrojejunostomy or other by-pass procedures, or endoscopically applied self-expanding metallic stents. However, some uncontrolled case series do suggest better survival for selected patients undergoing resection of lung or liver metastases or surgical removal of Krukenberg tumors; currently surgery of metastases remains experimental, however, until further evidence (Shiono 2013, Rosa 2016, Markar 2017). Similarly, few Asian studies have proposed a notable survival benefit for adjuvant hyperthermic intraperitoneal chemotherapy (HIPEC) in high-risk curatively resected gastric cancer patients (Fujimura 1994, Fujimoto 1999). Cytoreductive surgery together with HIPEC is also studied as a treatment for patients with advanced peritoneal metastases trying for a survival benefit (Glehen 2010, Yang 2011). Currently in Europe, HIPEC in treatment of gastric cancer is used only in the context of clinical research.

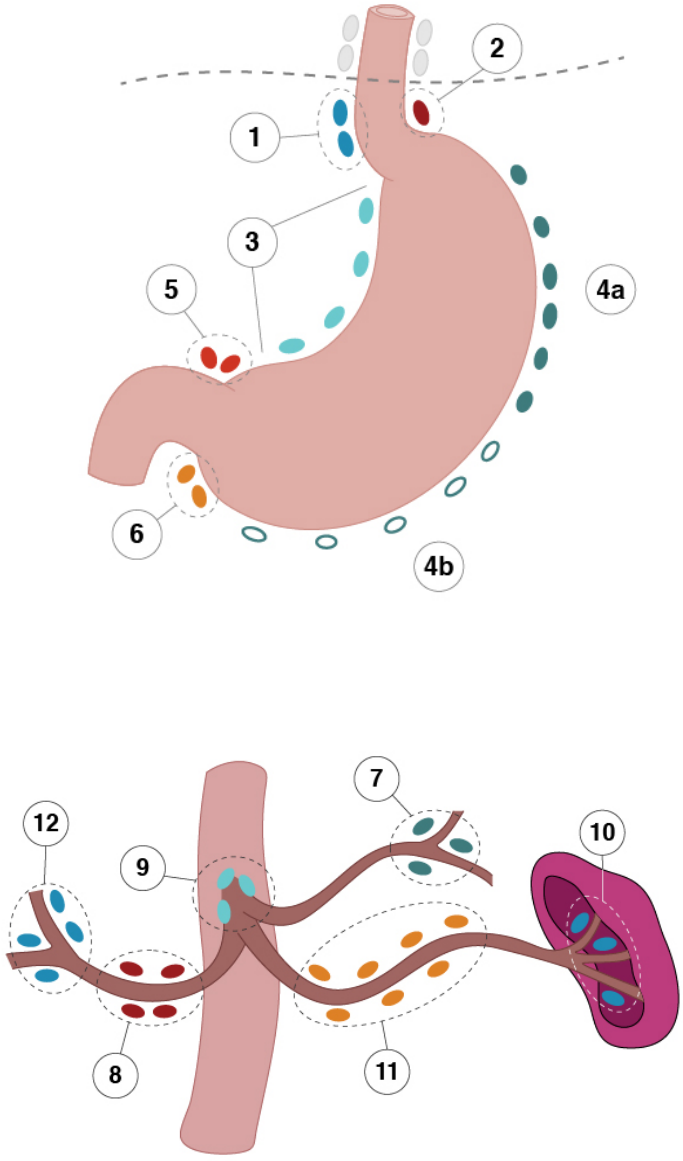


Figure 4. Regional lymph nodes of the stomach: right (1) and left (2) paracardial nodes, perigastric nodes of the lesser (3) and the greater (4a, 4b) curvatures, suprapyloric (5) and infrapyloric (6) nodes, nodes along the left gastric (7), the common hepatic (8), and the celiac (9) arteries, nodes of splenic hilum (10), nodes along the splenic artery, and hepatoduodenal (12) nodes. Figure drawn by Marja Ojala.

6.6.2 Oncological treatment

Surgeons have tried to improve the prognosis of gastric cancer by extending lymph node dissection in radical surgery, but without improved results. It is obvious that better survival can be achieved only by finding effective pre- and postoperative oncological modalities.

The present European guidelines recommend perioperative (pre- and postoperative) chemotherapy with a platinum/fluoropyrimidine combination for patients with stage IB or advanced resectable gastric cancer (Smyth 2016). This recommendation is based on randomized trials. The MAGIC trial showed a survival benefit from 23% to 36% in 5-year survival for patients treated with six cycles of perioperative chemotherapy (three pre- and three postoperative) in resectable stage II and III gastric cancer compared with surgery alone (Cunningham 2006). Another study has demonstrated a similar result, but a majority of the patients included, had proximal tumors, comprising cancers of the EGJ (Ychou 2011).

If gastric cancer has been operated on directly without preoperative chemotherapy, and is stage IB or advanced, postoperative chemoradiotherapy or adjuvant chemotherapy is the recommendation (Smyth 2016). Earlier, postoperative chemoradiotherapy was standard treatment, based on a trial that showed improved overall survival benefit compared to that of surgery alone (Macdonald 2001, Smalley 2012). Lack of adequate lymphadenectomy has inspired criticism of the trial, suggesting that the benefit of postoperative chemoradiotherapy may only compensate for this suboptimal surgery (Smyth 2016). The Dutch D1D2 trial also showed retrospectively that after D1 dissection, postoperative chemoradiotherapy improved survival, but not after optimal D2 resection (Dikken 2010). However, other studies also support postoperative chemoradiotherapy even after adequate surgery; this subject is under debate and requires further investigation (Kim 2005, Zhu 2012, Park 2015). The survival benefit of postoperative adjuvant chemotherapy has been demonstrated mainly in Asian studies (Sakuramoto 2007, Sasako 2011, Bang 2012, Noh 2014). A large, international meta-analysis of adjuvant chemotherapy confirmed a 6% benefit for chemotherapy compared with surgery alone (GASTRIC Group 2010).

A notable number of gastric cancer patients are diagnosed with already inoperable locally advanced or metastatic disease. Chemotherapy is the treatment of choice for them if co-morbidities, organ function, and performance status allow (Smyth 2016). Chemotherapy has improved survival and quality of life compared with results from supportive care only (Glimelius 1997, Bouché 2004). About 10% to 15% of gastric cancers overexpress human epidermal growth factor receptor 2 (HER2). Trastuzumab, a monoclonal antibody against HER2, is recommended for those patients with advanced disease as a target treatment, in combination with chemotherapy, for improved survival (Bang 2010, Smyth 2016).

6.6.3 Early gastric cancer (EGC) and endoscopic techniques

EGC is confined to the mucosa or submucosa without lymph node metastases; it is more often discovered in Asian countries because of their more comprehensive screening programs. When it is more readily identified and treated, survival rates are correspondingly much better. Patients with EGC have an even more favorable prognosis after radical surgery, and because lymph node metastasis is relatively infrequent, less invasive surgery may be practical (Tanizawa 2010). Endoscopic resection techniques may be an option for curative treatment for patients with intestinal-type cancer less than 2 cm in diameter without submucosal invasion or lymph-angio invasion. Risk of lymph-node metastases in this group is minimal (Nakajima 2002, Smyth 2016). Careful preoperative staging, correct patient selection, and an accurate report by an experienced pathologist are required for successful resection. Endoscopically treated patients have shown a disease-specific survival at 5 years of more than 95% (Bennett 2009).

The two forms of endoscopic resection are endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). EMR is suitable for lesions smaller than 10 to 15 mm with a polypoid or elevated form. However, ESD is the treatment of choice for most gastric superficial neoplastic lesions by European Society of Gastrointestinal Endoscopy Guidelines (Pimentel-Nunes 2015).

6.7 Prognostic factors

6.7.1 TNM classification

The most important factors that determine the prognosis of a patient with gastric cancer are radical surgery with adequate lymph-node dissection and stage of the disease at diagnosis. The Union for International Cancer Control (UICC)/the American Joint Committee on Cancer (AJCC) guidelines and their staging manual's tumor-node-metastasis (TNM) system is the most widely used and accepted staging classification system, continously evolving because of periodic validation studies. At present, the seventh edition has been in clinical practice (Table 1 and 2, Figure 5) but the eighth edition is already published (Sobin 2009, Edge 2010, Brierley 2017). The classification recommends dissection of a minimum of 15 lymph nodes to allow reliable staging.

Table 1. TNM classification of gastric cancer. Adapted from TNM Classification of Malignant Tumours, 7th Edition (Sobin 2009).

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria, high grade dysplasia
T1	Tumor invades lamina propria, muscularis mucosae (**T1a**), or submucosa (**T1b**)
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa
T4	Tumor perforates serosa (**T4a**) or invades adjacent structures (**T4b**)
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-2 regional lymph nodes
N2	Metastasis in 3-6 regional lymph nodes
N3	Metastasis in 7 or more regional lymph nodes (**N3a**: 7-15, **N3b**: 16 or more)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 2. TNM staging and 5-year survival for surgically resected gastric cancers. Adapted from TNM Classification of Malignant Tumours, 7th Edition and AJCC Cancer Staging Manual (Edge 2010, Sobin 2009).

Stage	T	N	M	5-year survival (%)
0	Tis	0	0	
IA	1	0	0	70.8
IB	2	0	0	57.4
	1	1	0	
IIA	3	0	0	45.5
	2	1	0	
	1	2	0	
IIB	4a	0	0	32.8
	3	1	0	
	2	2	0	
	1	3	0	
IIIA	4a	1	0	19.8
	3	2	0	
	2	3	0	
IIIB	4b	0,1	0	14.0
	4a	2	0	
	3	3	0	
IIIC	4a	3	0	9.2
	4b	2,3	0	
IV	Any	Any	1	4.0

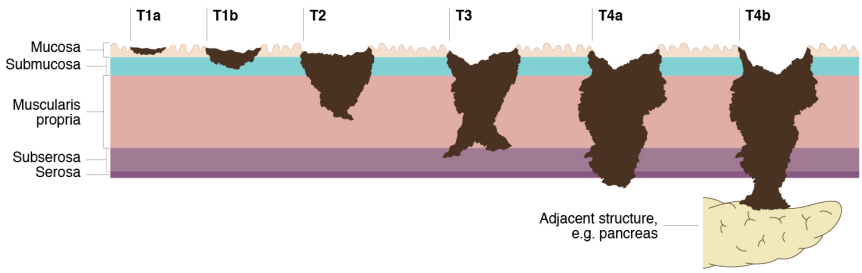


Figure 5. The extent of tumor (T) in TNM classification of gastric cancer (Sobin 2009). Figure drawn by Marja Ojala.

6.7.2 Tumor location and histology

Proximally located tumors tend to have a worse prognosis than distal tumors. The lesser curve of the stomach harbors more gastric cancer tumors than does the greater curve. In addition, diffuse-type cancers typically have more peritoneal metastases, whereas the intestinal type favors blood-borne metastases. The diffuse type tends to develop metastases early and is associated with poor outcome (Laurén 1965, Archie 2006).

6.7.3 Biomarkers

Besides the early detection and primary prevention of gastric cancer, the key to improving patient outcome may arise from finding more effective treatments and personalizing individual patient treatment based on prognostic and response-predictive factors such as biomarkers.

In clinical practice, human epidermal growth factor receptor 2 (HER2) is the only predictive biomarker for targeted therapy currently used for patient selection in gastric cancer. The mean frequency of HER2 overexpression in gastric and gastroesophageal cancer is 18%, and it is more common in intestinal type cancer. Studies indicate that positive HER2 is a negative prognostic factor, predicting more aggressive biological behavior and higher frequencies of recurrence (Tanner 2005, Abrahao-Machado 2016). Trastuzumab, a monoclonal antibody direct against HER2, was one of the first developed molecular-targeted drugs. It was first introduced for the treatment of HER2-positive advanced breast cancer. In HER2-expressing unresectable gastric and gastroesophageal cancers, trastuzumab together with chemotherapy causes an increase in overall survival compared to chemotherapy alone (Bang 2010).

The most extensively studied tumor markers in gastric cancer are serum carcinoembryonic antigen (CEA), and carbohydrate antigens 19-9 (CA 19-9) and 72-4 (CA 72-4). None of them is sensitive enough to stand alone as indicators of the presence of the disease or nor does either of them predict survival (Kodera 1996, Lai 2002, Huang 2014, Shimada 2014).

6.8 Biomarkers in this thesis

6.8.1 PODXL

Podocalyxin (PODXL) is a cell surface transmembrane protein belonging to the CD34 family, which is encoded on chromosome 7q32-q33. PODXL was first described in kidney podocytes as an anti-adhesive protein. It is a major component of the cell coat, glycocalyx of the glomerular podocytes, and thus this molecule was called podocalyxin (Kerjaschki 1984). PODXL is also expressed in vascular and

breast endothelium, in hematopoietic progenitors, and it is involved in neural development (Horvat 1986, Kerosuo 2004, Somasiri 2004, Vitureira 2010).

PODXL expression, reported in various cancers, has mostly been linked to poor prognosis, for example in breast, bladder, pancreatic, colorectal, and esophageal cancers, and in glioblastoma multiforme (Somasiri 2004, Larsson 2011,2012, Binder 2013, Boman 2013, Kaprio 2014, Heby 2015, Saukkonen 2015, Borg 2016). To the best of our knowledge, no earlier studies concern PODXL expression in gastric cancer.

The role of PODXL in tumorigenesis and cancer is not widely understood. PODXL has been thought to promote cancer cell invasion and migration, thus enhancing metastatic potential (Lin 2014, Flores-Téllez 2015, Snyder 2015). Other mechanisms are PODXL's evading the immune response by serving as an immunomodulatory molecule and maintaining and regulating glucose-transporters' surface expression (Schopperle 2010, Amo 2015). Interestingly, in cell lines of osteosarcoma, PODXL has shown resistance to cisplatin, an important cytotoxic drug also used in the treatment of gastric cancer (Huang 2015).

6.8.2 PROX1

Prospero homeobox protein 1 (PROX1) is a transcription factor that binds DNA and promotes transcriptional regulation of other genes. It belongs to a family of homeobox transcription factors, and the gene is localized on chromosome 1q32.2–q32.3. PROX1 protein contains 737 amino acids with a molecular mass of 82.3 kDa (Zinovieva 1996, Elsir 2012). PROX1 has appeared as a key regulatory protein in neurogenesis and organ development. It is important in embryonic development of the lens, retina, liver, pancreas, and lymphatic vasculature; PROX1 knockout mice have multiple developmental defects leading them to die before birth (Oliver 1993, Wigle 1999, Elsir 2012).

As a transcriptional regulator, varying levels of PROX1 expression have been reported in several cancer types, with the role of PROX1 apparently varying from tumor-suppressive to oncogenic. PROX1 is able to both activate and inhibit transcription of genes, and in many cancers, what is unclear is whether the role of PROX1 lies more in tumor initiation or in progression (Abate-Shen 2002, Elsir 2012).

Varying levels of PROX1 protein occur in various cancers, and its clinical significance is controversial depending on the cancer tissue. PROX1 expression has been associated with inferior patient outcome and cancer progression in colorectal and hepatocellular cancers, and in malignant gliomas (Shimoda 2006, Petrova 2008, Elsir 2010, Skog 2011, Liu 2013). Other malignancies that PROX1 is involved in included neuroblastoma, breast cancer, pancreatic cancer, esophageal cancer, carcinomas of the biliary system, hematologic malignancies, and Kaposi's sarcoma

(Nagai 2003, Schneider 2006, Laerm 2007, Versmold 2007, Yoshimoto 2007, Yoo 2010, Foskolou 2013, Saukkonen 2016).

In gastric cancer, PROX1 may play a role in tumor progression by enhancing cancer-cell proliferation and lymphangiogenesis, serving as a potential prognostic factor and target for treatment (Park 2017). Park et al. also studied the prognostic role of PROX1 in gastric cancer patients by immunohistochemistry, finding that the prognosis with PROX1-positive tumors was significantly worse than with negative tumors. In addition, dysregulation of microRNAs (miRNAs) are linked to tumorigenesis and tumor progression, and miR-489 has been downregulated in gastric cancer tissue. PROX1 is a direct miR-489 target serving, for this miR-489/PROX1 axis, as a potential therapeutic target in gastric cancer (Zhang 2016).

6.8.3 UCHL5

In the cell, the proteasome plays an important role in proteostasis by carrying out controlled protein degradation. Ubiquitin carboxyl-terminal hydrolase L5 (UCHL5), also called ubiquitin C-terminal hydrolase 37 (Uch37), is a cysteine protease belonging to the family of ubiquitin C-terminal hydrolases. It is one of three known human proteasome-associated deubiquitinating enzymes (DUBs), with a molecular mass of 37 kDa; it consists of 329 amino acids (Yao 2006, Jiao 2014). UCHL5 binds to its proteasome subunit Admr1/Rpn13 via reversible association, which activates its DUB activity (Matilainen 2013, Tian 2014). The function of UCHL5 is crucial, as UCHL5 knockout in mice is embryonically lethal (Al-Shami 2010).

In human tissues, expression level and cellular location of UCHL5 vary. It occurs in both healthy and cancerous tissues and has been associated with Alzheimer's disease and pulmonary fibrosis (Kikuchi 2013, Nan 2016). Proteasome inhibitors, for example bortezomib, serve as therapeutics for refractory multiple myeloma and mantle cell lymphoma (Schmidt 2014, Selvaraju 2015).

The ubiquitin-proteasome system, and UCHL5 as a part of it, is linked to cancer partly due to its capability to regulate many cell cycle proteins and apoptotic molecules (Mani 2005, Kitagawa 2009). High UCHL5 expression associates with cancer recurrence and poor survival in esophageal squamous cell, hepatocellular, and epithelial ovarian cancers (Chen 2012, Fang 2013, Wang 2014). In contrast, patients with high UCHL5 expression in pancreatic ductal adenocarcinoma and lymph-node-positive rectal cancer tend to have better prognosis (Arpalahti 2017). To the best of our knowledge, no studies concerning UCHL5 and gastric cancer exist.

6.8.4 MMP-8

Matrix metalloproteinase-8 (MMP-8) is part of the genetically distinct but structurally similar family of zinc-dependent metalloendopeptidases. Up to now, 24 different vertebrate MMPs have been recognized, of which 23 are identified in

humans. MMPs play an important role in many biological processes, such as embryogenesis, tissue remodeling, wound healing, and angiogenesis. MMPs can be classified based on their primary structures and substrate specificities. The key feature of MMP-8 enzyme, also called collagenase-2, is its ability to cleave interstitial collagens and other molecules. MMP-8 is mainly produced by neutrophils and encoded on chromosome 11q21-q22 (Nagase 1999, Egeblad 2002, Visse 2003). Elevated MMP-8 levels have been detectable in different inflammatory diseases such as periodontitis and *H.pylori* gastritis, and also in cardiovascular diseases (Sorsa 2004, Tuomainen 2007, Rautelin 2009, Pradhan-Palikhe 2010).

The extracellular matrix (ECM) of tumors and the non-cancerous, stromal cells within tumors also have an effect on tumor progression (Bissell 2001). MMPs have an ability to hydrolyze components of the ECM and also significantly to influence in all six steps, or hallmarks, of cancer development by promoting the growth and survival of cancer cells, regulating invasion by degrading structural ECM, by promoting angiogenesis and the epithelial-to-mesenchymal transition (EMT), and by inhibiting immune reactions against cancer cells (Hanahan 2000, Egeblad 2002). Increased expression of certain MMPs have been detectable in various cancer types, and their over-expression is often associated with poor prognosis. Some synthetic pharmaceutical MMP inhibitors have been developed for anticancer drugs, but the results in clinical trials have been disappointing because of major adverse effects or lack of benefits (Egeblad 2002).

The role of MMP-8 in cancer is more complex. MMP-8 may have protective properties in cancer through its capability to regulate the inflammatory response (Balbín 2003). In tongue cancer, MMP-8 has shown antitumor activity, and in breast cancer it may protect against lymph-node metastasis (Decock 2008, Korpi 2008, Soria-Valles 2014). In contrast, in hepatocellular carcinoma, in melanoma, and in colorectal cancer, increased levels of MMP-8 have been associated with an unfavorable course of the disease (Vihinen 2008, Väyrynen 2012, Lempinen 2013).

6.8.5 TIMP-1

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a specific inhibitor that binds MMPs in 1:1 stoichiometry. In vertebrates, thus far four different TIMPs have been identified, and they are expressed during development and tissue remodeling at various levels. TIMP-1 is capable of inhibiting all known MMPs except MT1-MMP, and has a crucial function in maintaining a balance between ECM deposition and degradation under differing physiological conditions (Will 1996, Gomez 1997, Brew 2000). When MMP activities are unbalanced under pathological conditions, varying levels of TIMPs are considered important, because of their ability to directly affect MMP activity levels (Visse 2003).

Despite the major function of TIMP-1 as an inhibitor of MMPs, it takes part in tumor invasion and metastasis in a more complex, and sometimes even paradoxical, way.

Because overexpression of MMPs is considered to promote tumor progression, it would be expected that elevated levels of TIMP-1 would then inhibit this process. Paradoxically, several studies, first in colorectal cancer, have associated elevated levels of TIMP-1 with the most aggressive tumors and worse prognosis (Urbanski 1993, Zeng 1995). Investigations have suggested that TIMP-1 may have other proteinase-independent activities, including upregulation of anti-apoptotic proteins and vascular endothelial growth factors, affecting tumor angiogenesis and direct stimulation of cell proliferation (Hayakawa 1992, Yoshiji 1998, Egeblad 2002, Jiang 2002, Kessenbrock 2010).

High levels of TIMP-1 seem to correlate with poor prognosis in many cancers, so TIMP-1 has been under study as a potential prognostic biomarker, for example in breast and colon cancers, but it is not yet in clinical use (Egeblad 2002, Schrohl 2004, Würtz 2008, Birgisson 2010). In gastric cancer, several studies consider TIMP-1 as a prognostic biomarker for worse prognosis (Joo 2000, Yoshikawa 2001). Grunnet et al. reviewed TIMP-1 in gastric cancer and found 17 articles fulfilling the criteria; they concluded that elevated levels of TIMP-1 protein in either tumor tissue or in plasma associates with reduced survival in gastric cancer (Grunnet 2013).

7 AIMS OF THE STUDY

The purpose of the study was to evaluate the expression and prognostic significance of potential biomarkers in gastric cancer.

The specific aims were to study the prognostic value of

- PODXL immunohistochemical expression in relation to clinicopathological parameters by two different antibodies.
- PROX1 immunohistochemical expression in relation to clinicopathological parameters.
- UCHL5 immunohistochemical expression in relation to clinicopathological parameters.
- MMP-8 serum levels and immunohistochemical expression in relation to clinicopathological parameters.
- TIMP-1 serum levels in relation to clinicopathological parameters.

8 PATIENTS AND METHODS

8.1 Patients (I-IV)

A total of 650 gastric cancer patients underwent surgery for histologically verified gastric adenocarcinoma at the Department of Surgery, Helsinki University Hospital between 1983 and 2009. These studies are based on two tissue microarray (TMA) series. The first TMA series includes tissue samples from 337 patients operated on between 1983 and 1999 and is the material in Study I. The second TMA series includes 313 patients operated on from 2000 to 2009 and is the material in Studies II and IV. In Study III, both series (650) were combined.

The clinicopathological characteristics of the two study populations are presented in Table 3. Overall, 371 (57.1%) were operated on for curative intent, whereas 261 (40.2%) underwent palliative surgery. Data on cancer resectability was missing in 18 (2.7%) cases. Extended lymphadenectomy (D2-D2+) was done for 237 (36.5%). Preoperative treatment received 15 (2.3%), 157 (24.2%) received post-operative adjuvant treatment (101 chemotherapy, 4 radiotherapy, and for 52 both). Median age was 66.9 (interquartile range (IQR) 57.0-75.0). Median follow-up time was 1.6 years (IQR 0.6-4.7). The 5-year overall survival rate for the whole cohort was 36.2% (95% confidence interval (CI) 31.7-40.7%).

Survival data and cause of death for Studies I-IV came from patient records, the Population Register Centre of Finland, and Statistics Finland. The studies were approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013), and the National Supervisory Authority of Welfare and Health gave permission to use the tissue samples without individual consent in these retrospective studies (Valvira Dnro 10041/06.01.03.01/2012).

Table 3. Clinicopathological characteristics of the two study populations.

	Study population 1983–1999	Study population 2000–2009
n (%)	**337**	**313**
Age, years		
< 66	165 (49.0)	148 (47.3)
≥ 66	172 (51.0)	165 (52.7)
Gender		
Male	174 (51.6)	152 (48.6)
Female	163 (48.4)	161 (51.4)
Stage		
I	100 (29.7)	62 (19.8)
II	41 (12.2)	72 (23.0)
III	96 (28.5)	115 (36.7)
IV	100 (29.7)	63 (20.1)
Primary tumor, T		
T1	59 (17.5)	49 (15.7)
T2	61 (18.1)	44 (14.1)
T3	154 (45.7)	98 (31.3)
T4	63 (18.7)	122 (39.0)
Lymph node metastases, N		
N0	152 (45.1)	104 (33.2)
N1	95 (28.2)	44 (14.1)
N2	89 (26.4)	72 (23.0)
N3		82 (26.2)
Distant metastases, M		
M0	244 (72.4)	250 (79.9)
M1	93 (27.6)	63 (20.1)
Laurén classification		
Intestinal	142 (42.1)	124 (39.6)
Diffuse	195 (57.9)	179 (57.2)
Tumor size, cm		
< 5	185 (54.9)	115 (36.7)
≥ 5	146 (43.3)	190 (60.7)

8.2 Tumor tissue specimens (I-IV)

Formalin-fixed and paraffin-embedded tumor samples were collected from the archives of the Department of Pathology, Helsinki University Hospital. The patient tissues were de-identified and analyzed anonymously. An experienced pathologist marked representative tumor areas for microarrays on hematoxylin- and eosin (H&E)-stained slides. In the first TMA series (Studies I and III) three 0.6-mm cores and in the second TMA series (Studies II-IV) four 1.0-mm cores were punched with

a semiautomatic tissue microarray instrument (Beecher Instruments, Silver Spring, MD, USA). The cores were embedded in paraffin as tissue-array blocks (Kononen 1998).

8.3 Immunohistochemistry (I-IV)

The tumor tissue microarrays blocks were freshly cut into 4-µm thick sections, fixed on slides, and dried for 12 to 24 hours at 37°C. After deparaffinization in xylene, and rehydration through a gradually decreasing concentration of ethanol to distilled water, slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in antibody-specific buffer for 20 minutes at 98°C for antigen retrieval. Section staining was performed in an Autostainer 480 (Lab Vision) by the Dako REAL EnVision Detection system, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). Slides were incubated with the chosen antibody for one hour at room temperature. For a descriptive list of antibodies used (I-IV) see Table 4.

In Study I, we used two PODXL antibodies against different epitopes. The polyclonal antibody (HPA2110, Atlas Antibodies, Stockholm, Sweden) recognizes amino acid residues 278–415 of PODXL, and the monoclonal in-house antibody HES9 (produced by our collaborators at Fujirebio Diagnostics Ab, Gothenburg, Sweden) recognizes the amino acid residues 189–192 of PODXL. Both of these epitopes are located in the extracellular part of PODXL (Uhlén 2005, Pontén 2008, Kaprio 2014).

Table 4. Antibodies for immunohistochemistry

Antibody	Company	Dilution	Control tissue	Study
PODXL HES9 mAb	In-house	1:500	Kidney	I
PODXL HPA2110 pAb	Atlas Antibodies	1:250	Kidney	I
PROX1 pAb	R&D Systems	1:1800	Colon	II
UCHL5 pAb	Sigma Aldrich	1:800	Colon	III
MMP-8 pAb	In-house	1:400	Colon, breast	IV

Abbreviations: mAb = monoclonal antibody, pAb = polyclonal antibody

8.4 Scoring of samples (I-IV)

Tumor specimens were scored independently by two, and in Study III, by three researchers blinded to clinical status and outcome data. Samples with discordant scores were re-evaluated until consensus. There were three (Studies I and III) or four (Studies II-IV) distinct tumor cores per patient, with the highest score of each sample serving for further analysis. All antibodies (I-IV) stained mainly cytoplasmic in gastric cancer cells, with their intensity of staining graded from 0 to 3. Negative

immunoreactivity was scored as 0, weakly positive as 1, moderately positive as 2, and strongly positive as 3.

8.5 Serum samples (IV)

Blood samples, in total from 233 patients, were collected within 24 days prior to the gastric cancer surgery. The majority of the samples (95.7%) were taken within 3 days (range 0-24 days) before the operation. The blood samples were centrifuged, and plasma and serum components stored as aliquots at -80°C until analysis. Serum MMP-8 concentrations were determined by time-resolved immunofluorometric assay (IFMA) (Medix Biochemica, Espoo, Finland) according to manufacturer's instructions; the detection limit for MMP-8 was 0.08 ng/ml (Tuomainen 2007). Serum levels of TIMP-1 were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Biotrak ELISA System; Amersham Biosciences, Buckinghamshire, UK) with a detection limit of 1.25 ng/ml (Lauhio 2016). For the calculation of MMP-8/TIMP-1 molar ratios, concentrations (ng/ml) were converted to molarities (mol/l) by use of the molecular weights of MMP-8 and TIMP-1 (Visse 2003).

8.6 Statistical analysis (I-IV)

Immunohistochemical expressions were dichotomized for statistical purposes: PODXL, UCHL5, and MMP-8 as negative (score 0) vs. positive (scores 1-3), and PROX1 as low (0-1) vs. high (2-3) immunostaining. Associations between various immunoexpression and clinicopathological variables were assessed by the chi-square and Fisher's exact tests. Correlations between the two PODXL antibodies (Study I) were assessed by Spearman's correlation test. In Study IV, the Mann-Whitney U-test and Kruskal-Wallis test allowed determination of the significance of difference in biomarker median serum concentrations among gastric cancer subgroups. For serum biomarkers MMP-8 and TIMP-1 and for the MMP-8/TIMP-1 molar ratio (Study IV), we determined optimal cut-offs by the aid of receiver-operating characteristic (ROC) curves and found them to identify groups suitable for survival analyses. Cancer-specific survival was calculated from date of surgery to death from gastric cancer or until follow-up. Patients who died from causes other than gastric cancer were censored at the date of their death. Survival curves were constructed according to the Kaplan-Meier method and compared with the log-rank test. Uni- and multivariate survival analyses were performed with the Cox proportional hazard model according to the backward stepwise method. All statistical tests were two-sided. A p-value below 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 20.0-25.0 software (IBM SPSS Statistics, Chicago, IL, USA).

9 RESULTS

9.1 Immunohistochemistry (I-IV)

The score distribution by the various antibodies studied is presented in Table 5.

In Study I, both PODXL antibodies stained evenly throughout the cytoplasm without nuclear nor cell membranous staining. Weak to strongly positive scores (1-3) were regarded as positive expression for the following analysis. Expression of PODXL by these two different antibodies correlated (r_s=0.455, p<0.001, Spearman's rank correlation test).

In Study II, cytoplasmic PROX1 expression was negative or weakly positive in 217 (79.5%) cases and was regarded as low expression for final analysis. The other group, high expression, included 56 (20.5%) moderately or strongly positive tumor samples. The staining occurred mainly in the cytoplasm, but also, in some strongly stained samples, a little nuclear immunopositivity was detectable.

In Study III, cytoplasmic and nuclear UCHL5 staining occurred, but due to an overlap in a large number of samples, no separate evaluation of nuclear staining was possible. Cytoplasmic UCHL5 expression was negative in 111 (22.7%) and positive in 379 (77.3%) samples.

In Study IV, MMP-8 immunoexpression was also cytoplasmic, and neutrophils also showed MMP-8 immunopositivity. MMP-8 expression was negative in 157 (56.9%) and positive (scores 1-3) in 119 (43.1%) cases.

Table 5. Score distribution of immunohistochemical markers in Studies I-IV.

Tumor marker	Cytoplasmic expression score, n (%)				Total
	0	1	2	3	
PODXL HES9	67 (24.0)	137 (49.1)	54 (19.4)	21 (7.5)	279
PODXL HPA2110	113 (42.5)	120 (45.1)	29 (10.9)	4 (1.5)	266
PROX1	118 (43.2)	99 (36.3)	39 (14.3)	17 (6.2)	273
UCHL5	111 (22.7)	217 (44.3)	119 (24.3)	43 (8.8)	490
MMP-8	157 (56.9)	85 (30.8)	30 (10.9)	4 (1.4)	276

9.2 Association with clinicopathological characteristics (I-IV)

Associations of different biomarkers with clinicopathologic characteristics were analyzed by the chi-square test.

In Study I, positive PODXL staining by both antibodies (HPA2110 and HES9) associated with intestinal cancer type ($p<0.001$ for both). Positive HES9 staining also associated with age 66 or over ($p=0.001$) and with small-sized (≤ 5 cm) tumors ($p=0.024$, Table 6).

In Study II, low PROX1 immunostaining associated with diffuse cancer type ($p=0.002$, Table 6).

In Study III, positive immunostaining of UCHL5 associated with intestinal cancer type ($p=0.004$, Table 6), but not with any other parameters studied.

In Study IV, negative MMP-8 immunoexpression associated with patient under 67 ($p=0.007$), stage I cancer ($p=0.022$), tumor classification T1 ($p=0.005$), cancer without lymph node metastasis ($p=0.016$), and with diffuse cancer type ($p<0.001$, Table 6).

Table 6. Association of tissue biomarkers with clinicopathological variables, NS= not significant ($p\geq0.05$).

	PODXL HPA2110	PODXL HES9	PROX1	UCHL5	MMP-8
Age	NS	p=0.001	NS	NS	p=0.007
Gender	NS	NS	NS	NS	NS
TNM stage	NS	NS	NS	NS	p=0.022
pT-classification	NS	NS	NS	NS	p=0.005
pN-classification	NS	NS	NS	NS	p=0.016
pM-classification	NS	NS	NS	NS	NS
Laurén classification	p<0.001	p<0.001	p=0.002	p=0.004	p<0.001
Tumor size	NS	p=0.024	NS	NS	NS

9.3 Serum MMP-8 and TIMP-1 results (IV)

Median MMP-8 serum level was 54.8 ng/ml (IQR 30.8-105 ng/ml) and for TIMP-1, 156 ng/ml (IQR 132-187 ng/ml). Median molar ratio of MMP-8/TIMP-1 was 0.153 (IQR 0.082-0.280). Serum levels of MMP-8 and TIMP-1 were higher in patients with the intestinal cancer type (p=0.044, p=0.021, Mann-Whitney U-test). TIMP-1 serum levels were also higher among patients over age 67 (p<0.001), with metastasized disease (p=0.035), and in samples with positive MMP-8 immunohistochemistry (p=0.008). Moreover, the MMP-8/TIMP-1 molar ratio was higher among patients under 67 years (p=0.034, Table 1 in Study IV).

9.4 Survival analysis (I-IV)

In Study I, Kaplan-Meier analysis showed significantly worse cancer-specific survival for patients with positive PODXL expression. Gastric cancer-specific 5-year survival, by polyclonal antibody HPA2110, was 24% (95% CI 16.9-31.1) for positive expression, compared to 43% (95% CI 33.7-52.9) for patients with negative expression (p=0.001 log-rank test, Figure 6, Table 7). The 5-year survival rate of patients with PODXL-positive tumors by monoclonal HES9 antibody was 30% (95% CI 23.1-36.1), and with negative expression, 40% (95% CI 27.7-52.1; p=0.130 log-rank test, Table 7). Positive PODXL was a marker of worse prognosis in the subgroups of younger (< 66 years) patients (p=0.006), for men (p=0.002), diffuse cancer (p=0.001), and TNM stage I (p=0.048).

In Study II, the gastric cancer-specific survival for patients with high PROX-1 expression was significantly better than for patients with low immunoexpression. The 5-year survival rate for patients with high expression was 65.6% (95% CI 52.7–78.5), compared to 37.1% (95% CI 30.2–44.0) for patients with low expression (p=0.004, log-rank test, Figure 7, Table 7). In subgroup analysis, high PROX1 expression was a marker of better prognosis in subgroups of younger (< 66 years) patients (p=0.007), men (p=0.019), patients with small (< 5 cm) tumors (p=0.030), and in the subgroup of intestinal cancer (p=0.025).

In Study III, no significant difference emerged in cumulative survival between patients with UCHL5-negative or -positive immunostaing. The 5-year cancer-specific survival rate for the negative group was 31.3% (95% CI 22.5-40.4), and 37.7% (95% CI 32.5-42.8) for the positive group (p=0.107, log-rank test, Table 7). Positive UCHL5 was a marker of better prognosis in the subgroups of patients aged 66 or older (p=0.037), in TNM stage I-II (p=0.025), and in patients with small (< 5cm) tumors (p=0.001).

In Study IV, for serum biomarkers MMP-8, TIMP-1, and the MMP-8/TIMP-1 molar ratio, we determined optimal cut-offs by the aid of receiver-operating characteristic (ROC) curves. The patients with serum MMP-8 lower than 31 ng/ml or over 131 ng/ml had a prognosis considerably worse than did patients with an intermediate (31-131 ng/ml) MMP-8 serum level (p=0.002, log-rank test, Figure 8A). The 5-year survival rate for patients with low serum MMP-8 was 29.7% (95% CI 17.2-42.2), 37.2% (95% CI 21.9-52.5) for high MMP-8 level, and 53.1% (95% CI 44.3-61.9) for intermediate level (Table 7). Patients with high (≥ 170 ng/ml) serum TIMP-1 concentration had a poor prognosis and a 5-year survival rate of 30.6% (95% CI 20.2-41.0) compared to those of patients with low (<170 ng/ml) level with a 5-year survival rate of 52.3% (95% CI 43.9-60.7) (p<0.001, log-rank test, Figure 8B, Table 7). The molar ratio of serum MMP-8/TIMP-1 had also two cut-offs, and patients with a low (< 0.07) or high (> 0.30) molar ratio had a worse prognosis than did those with an intermediate ratio (p=0.020, log-rank test, Figure 8C, Table 7). Differences in tissue MMP-8 immunoexpression had no significant influence on gastric-cancer-specific survival of patients (p=0.178, Table 7).

In subgroup analysis, intermediate MMP-8 serum level (31-131 ng/ml) was a marker of better prognosis in subgroups of patients aged 67 or over (p=0.015), in men (p=0.004), in TNM stages I-II (p=0.003), in pT2 (p<0.001) and pT3 (p=0.011) tumors, in lymph-node-positive cancers (p=0.037), in cancers without distant metastasis (p<0.001), in both intestinal (p=0.022) and diffuse (p=0.038) cancer, and in small (≤ 6 cm) tumors (p=0.001). Patients with low TIMP-1 concentration (< 170 ng/ml) had a better prognosis in the subgroups of both age categories: < 67 years (p=0.012) and ≥ 67 (p=0.038), in both genders: men (p=0.024) and women (p=0.003), in stages III-IV (p<0.001), in pT4 tumors (p<0.001), tumors with lymph-node metastasis (p<0.001), tumors both without (p=0.018) and with (p=0.016) distant metastasis, and in both intestinal (p=0.002) and diffuse (p=0.005) type cancers, and in both size categories: ≤ 6 cm (p=0.024) and > 6cm (p=0.002). Negative tissue immunostaining of MMP-8 was a marker of better prognosis in women (p=0.026) and among those with serum MMP-8 lower than 31 ng/ml (p=0.018). When MMP-8 immunostainings were analyzed as two subgroups: negative and positive, we found that intermediate serum MMP-8 level (31-131 ng/ml) was a significant marker of better prognosis in both subgroups. In addition, low TIMP-1 level (< 170 ng/ml) was a significant marker of better prognosis solely in the subgroup of MMP-8-positive immunostaining.

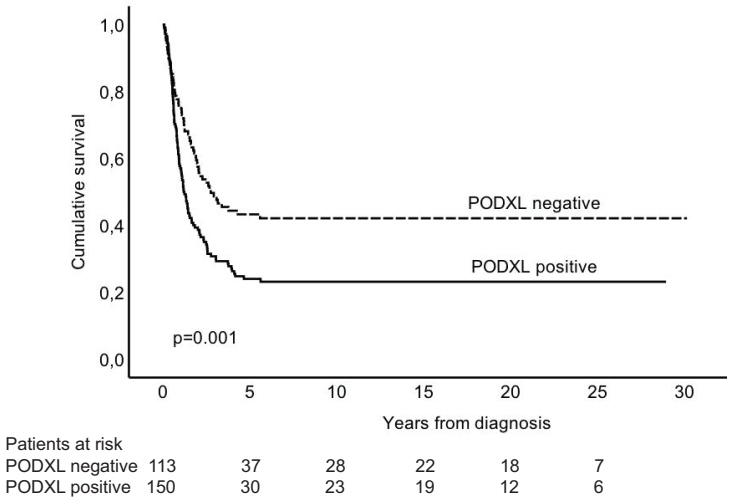


Figure 6. PODXL expression with polyclonal HPA2110 antibody and cancer-specific survival according to the Kaplan-Meier method, p-value for log-rank test.

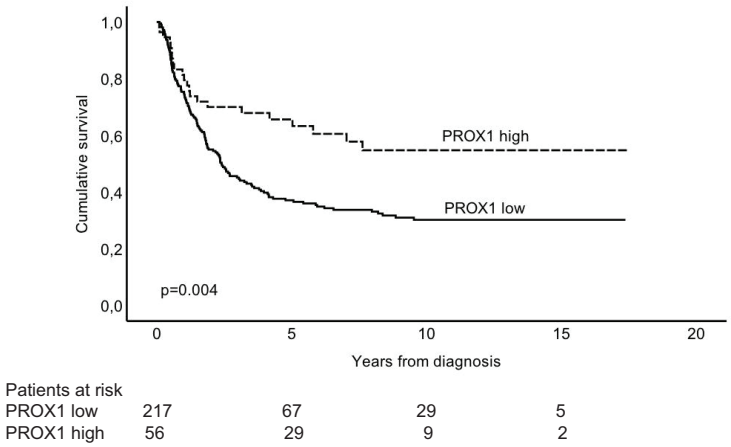
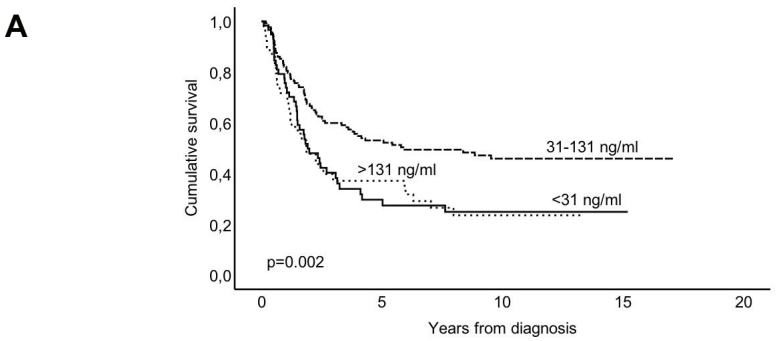
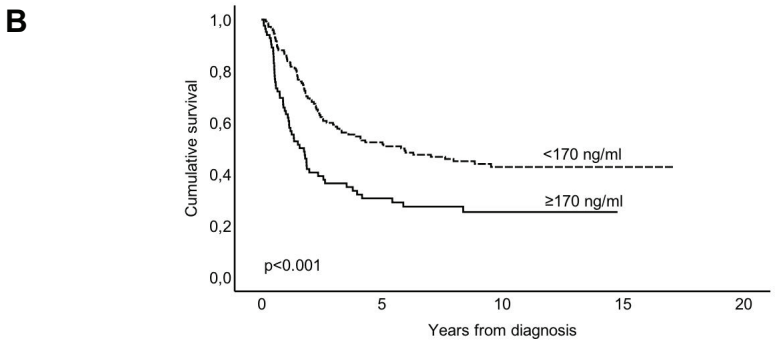


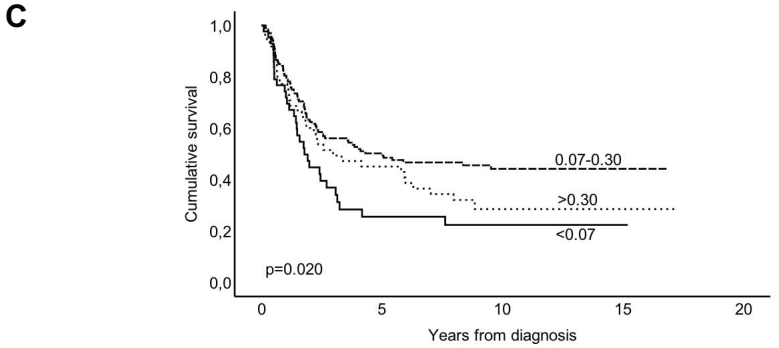
Figure 7. PROX1 expression and cancer-specific survival according to the Kaplan-Meier method p-value for log-rank test.



Patients at risk				
MMP-8 <31 ng/ml	60	13	8	1
MMP-8 31-131 ng/ml	133	62	31	5
MMP-8 >131 ng/ml	40	14	3	0



Patients at risk				
TIMP-1 <170 ng/ml	146	68	31	6
TIMP-1 ≥170 ng/ml	87	21	11	0



Patients at risk				
MMP-8/TIMP-1 <0.07	44	8	7	1
MMP-8/TIMP-1 0.07-0.30	139	60	28	4
MMP-8/TIMP-1 >0.30	50	21	7	1

Figure 8. Serum levels of A) MMP-8, B) TIMP-1, and C) MMP-8/TIMP-1 molar ratio and cancer-specific survival according to the Kaplan–Meier method, p-value for log-rank test.

Table 7. Kaplan-Meier analysis for cancer-specific survival (CSS) stratified for different clinicopathological variables and biomarkers in gastric cancer patients; p-value for log-rank test.

	5-year CSS %	95% CI	p-value
Gender			
Male	46.3	37.7-54.9	0.461
Female	40.9	33.1-48.7	
Age			
< 67	47.2	39.2-55.2	0.053
≥ 67	39.3	31.1-47.5	
Stage			
I	93.0	86.3-99.7	<0.001
II	64.5	52.5-76.5	
III	23.6	15.4-31.8	
IV	5.70	0-12.0	
Laurén classification			
Intestinal	52.0	42.6-61.4	0.020
Diffuse	37.9	30.7-45.2	
Tumor size			
≤ 6 cm	59.1	51.5-66.7	<0.001
> 6 cm	21.5	13.9-29.1	
PODXL HPA2110			
Negative	24.0	16.9-31.1	<0.001
Positive	43.3	33.7-52.9	
PODXL HES9			
Negative	30.0	23.1-36.1	0.130
Positive	40.0	27.7-52.1	
PROX1			
Low	37.1	30.2-44.0	0.004
High	65.6	52.7-78.5	
UCHL5			
Negative	31.3	22.5-40.4	0.107
Positive	37.7	32.5-42.8	
MMP-8			
Negative	46.3	38.1-54.5	0.178
Positive	36.3	27.1-45.5	
Serum MMP-8 (ng/ml)			
< 31	29.7	17.2-42.2	0.002
31-131	53.1	44.3-61.9	
> 131	37.2	21.9-52.5	
Serum TIMP-1 (ng/ml)			
< 170	52.3	43.9-60.7	<0.001
≥ 170	30.6	20.2-41.0	
Serum MMP-8/TIMP-1 molar ratio			
< 0.07	25.5	11.6-39.4	0.020
0.07-0.30	50.2	41.6-58.8	
> 0.30	45.1	30.8-59.4	

9.5 Multivariable survival analysis (I-IV)

Cox regression analysis for different clinicopathological variables and markers studied in the study population undergoing surgery between 2000 and 2009 is in Table 8. Age, stage, Laurén classification, tumor size, PROX1, and serum TIMP-1 level served as independent prognostic factors in multivariable survival analysis. Separate multivariable models were calculated in each study, utilizing current study populations and survival data.

In Study I, PODXL expression of the polyclonal antibody HPA2110 was a significant independent prognostic factor (hazard ratio (HR) 3.17, 95% CI 1.37–7.34, p=0.007). Other independent factors in multivariable analysis were tumor stage, grade, and DNA ploidy.

In Study II, PROX1 expression remained significant (HR 0.56, 95% CI 0.35–0.90, p=0.017) in multivariable analysis, together with patient age, metastasized disease, and tumor size.

In Study III, UCHL5 expression did not fulfill the Cox assumption of proportional hazard ratios over time with all patients included for a multivariable model. Nevertheless, UCHL5 expression was a significant prognostic factor in multivariable analysis in subgroups of patients at disease stages I-II (HR 0.35, 95% CI 0.19-0.65, p=0.001) and in patients with small (< 5 cm) tumors (HR 0.39, 95% CI 0.23-0.66, p<0.001).

In Study IV, high TIMP-1 serum level (≥ 170 ng/ml) was an independent prognostic factor (HR 1.85, 95% CI 1.26-2.72, p=0.002) in multivariable analysis. Patient age, TNM stage, and Laurén classification also served as independent factors. Serum level or tissue expression of MMP-8 were not performed by multivariable survival analysis.

Table 8. Cox regression analysis for cancer-specific survival of gastric cancer patients undergoing surgery between 2000 and 2009.

	Univariable survival analysis			Multivariable survival analysis		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Gender						
Male	1.00			1.00		
Female	1.12	0.83-1.50	0.461	1.04	0.70-1.56	0.842
Age						
< 67	1.00			1.00		
≥ 67	1.33	1.00-1.79	0.054	1.87	1.24-2.81	0.003
Stage						
I	1.00			1.00		
II	5.44	2.25-13.1	<0.001	4.75	1.57-14.3	0.006
III	15.7	6.85-36.1	<0.001	15.2	5.38-42.7	<0.001
IV	46.2	19.6-109	<0.001	60.0	20.1-179	<0.001
Laurén classification						
Intestinal	1.00			1.00		
Diffuse	1.45	1.06-1.98	0.020	1.68	1.09-2.59	0.018
Tumor size						
≤ 6 cm	1.00			1.00		
> 6 cm	2.71	2.00-3.68	<0.001	1.66	1.10-2.49	0.015
PROX1						
Low	1.00			1.00		
High	0.52	0.33-0.81	0.004	0.57	0.33-0.96	0.036
UCHL5						
Negative	1.00			1.00		
Positive	0.69	0.50-0.96	0.027	0.92	0.60-1.41	0.689
MMP-8						
Negative	1.00			1.00		
Positive	1.24	0.91-1.69	0.179	1.06	0.69-1.61	0.801
Serum MMP-8 (ng/ml)						
< 31	1.00			1.00		
31-131	0.56	0.38-0.84	0.004	0.69	0.44-1.09	0.114
> 131	0.92	0.57-1.48	0.724	1.03	0.59-1.78	0.927
Serum TIMP-1 (ng/ml)						
< 170	1.00			1.00		
≥ 170	1.93	1.37-2.72	<0.001	1.71	1.13-2.58	0.011

10 DISCUSSION

To the best of our knowledge, Studies I-IV show for the first time the prognostic significance of PODXL, PROX1, UCHL5, and MMP-8 expression in relation to clinicopathological variables in gastric cancer. In addition, TIMP-1 has proven to be a marker of poor prognosis, and we validated such a result in this gastric cancer cohort.

10.1 Biomarkers

10.1.1 PODXL

Study I showed PODXL to be an independent marker of unfavorable prognosis in gastric cancer, because patients with PODXL-negative tumors survived significantly better; actually, among stage-I patients with a PODXL-negative tumor, only one died from cancer. After our study, Borg et al. validated this result by also showing that PODXL is an independent marker of reduced survival in their TMA series of both gastric cancer and esophageal adenocarcinoma (Borg 2016).

The staining of the two antibodies, commercially available polyclonal antibody HPA2110 and in-house monoclonal antibody HES9, differed in their intensity. Both stained throughout the cytoplasm, but the staining intensity and distribution of the monoclonal antibody was stronger than that of the polyclonal antibody. PODXL is a transmembrane protein, but this TMA series of gastric cancer showed no staining in cell nuclei or cell membranes. The explanation for this cytoplasmic, non-membranous, expression is unknown. Borg et al. used the same polyclonal antibody, and the staining was also mainly in the cytoplasm, sometimes in a granular pattern, and they observed some strong membranous component in a few samples (Borg 2016). Membranous staining has been detectable and has served as a prognostic cut-off (membranous vs. non-membranous) at least in colorectal and pancreatic cancers (Larsson 2012, Heby 2015, Saukkonen 2015). In pancreatic cancer, the staining was membranous by both of these same antibodies that we used (Saukkonen 2015).

Results by use of these two antibodies were not identical, and case-by-case expressions differed. The antibodies are known to recognize different epitopes in the extracellular part of the PODXL molecule, and it is possible that they describe a distinct biological phases of PODXL explaining why their results differed in this study. Earlier, in colorectal cancer, these same two antibodies revealed an interesting finding, in which the polyclonal antibody stained membranously, whereas monoclonal antibody positivity was mainly cytoplasmic. Strong positivity of both antibodies revealed a subgroup of colorectal cancer patients with even worse prognosis (Kaprio 2014). In gastric cancer, we found no similar relationship. The

number of positive immunostainings was much higher among colorectal cancer patients than in this gastric cancer material (94% vs. 58%). In addition, the PODXL positivity in gastric cancer was close to that seen in breast (40%) and ovarian cancer (67%), and lower than in the other study, involving gastric cancer (78%) (Somasiri 2004, Cipollone 2012, Borg 2016). Several reasons could explain this discrepancy, for example, an observer-dependent explanation such as setting the cut-off between negative and weak-positive staining scores. Further studies should determine optimal prognostic cut-offs, which may, of course, differ among cancer types.

The function of PODXL in carcinogenesis is largely unknown. One theory is that PODXL enhances cancer cell invasion and migration, and promotes metastatic potential. Other theories propose its evasion of natural killer cell-mediated cytotoxicity and regulation of glucose transporter surface expression (Schopperle 2010, Lin 2014, Amo 2015, Flores-Téllez 2015, Snyder 2015). One in vitro study of gastric cancer tissues showed that migration and invasion abilities were tightly associated with PODXL expression. This offers a promising possibility to design a novel target agent that could block PODXL, resulting in inhibition of gastric cancer cell migration and invasion (Zhang 2017). In future, we need more research focused on the biological role of PODXL in various malignancies.

A recent systematic review and meta-analysis summarized studies related to the prognostic significance of PODXL expression among cancers. These 12 studies, comprising totally 5309 patients, concluded that high PODXL expression is an effective predictor of cancer and could be utilized as a promising prognostic biomarker (Wang 2016).

10.1.2 PROX1

Study II demonstrated that PROX1 is an independent marker of better prognosis in gastric cancer. This was the first study utilizing our new TMA series comprising gastric cancer tissue samples from patients operated on between 2000 and 2009 at Helsinki University Hospital. This series contains one additional tumor spot and the width of each spot is also greater than in our earlier TMA series.

PROX1 expression was visible throughout the cytoplasm, and some nuclear positivity was noted in a few strongly stained samples. Other studies have reported both cytoplasmic and nuclear immunostaining in gastric cancer (Taban 2014, Park 2017). Among cancers, staining pattern tends to differ. Nuclear staining is observable in colonic and hepatocellular cancers and in gliomas, whereas mainly cytoplasmic staining occurs in pancreatic cancer (Shimoda 2006, Elsir 2010, Skog 2011, Saukkonen 2016). The purpose of cytoplasmic expression is unknown. One theory is that PROX1 is enriched and activated in the cytoplasm before its translocation to the nucleus to become functionally active (Skog 2011).

As a transcription factor, PROX1 is a key regulatory protein in the development of various organs and is involved in many biological processes concerning cell-fate determination and progenitor-cell regulation. PROX1 may also exhibit tumor-suppressive or tumor-promoting effects, depending on tissue context, as is evident in several cancers studied. Positive PROX1 expression is associated with favorable prognosis, at least in pancreatic and hepatocellular cancers, and in carcinoma of the biliary system (Shimoda 2006, Laerm 2007, Saukkonen 2016). However, high PROX1 levels are associated in many cancer types with poor patient outcome, for example, in colorectal cancer, in rectal neuroendocrine tumors, and in renal cell carcinoma (Petrova 2008, Skog 2011, Lv 2014, Jernman 2015). This diversity of expression makes PROX1 an interesting and challenging molecule as a potential biomarker in cancers.

Few studies have concerned PROX1 in gastric cancer, and the results have been interestingly different from ours. Park et al. analyzed PROX1 by silencing its expression in gastric cancer cell lines and found this to inhibit cell proliferation. They suggested that PROX1 may regulate cell fate by reducing apoptosis as well as by promoting proliferation in gastric cancer cell lines. In the same study, they also studied the prognostic influence of PROX1 in gastric cancer patient samples by immunohistochemistry, finding that positive PROX1 expression associated with poor prognosis (Park 2017). Reasons for these contradicting results may be several. Their patient material differed from ours, as they did not include metastatic cancer at all, the antibodies were not the same, and the staining and scoring methods also differed from ours. Zhang et al. studied microRNA, specifically miR-489 in gastric cancer tissue and cell lines. They suggest that PROX1 is a direct target for miR-489, and PROX1 depletion would then suppress cell proliferation. Based on these findings, they hypothesized that low PROX1 expression would correlate with better patient prognosis (Zhang 2016). This fascinating discrepancy intrigues researchers considering on the role of PROX1 in gastric cancer and its effects on patient prognosis.

10.1.3 UCHL5

Positive UCHL5 expression revealed better survival in subgroups of stages I-II cancer, small tumor size (< 5cm), and age 66 or older. In our whole patient cohort of gastric cancer, the difference between positive and negative staining remained nonsignificant in survival analysis.

Our immunohistochemical staining pattern was mainly cytoplasmic, with some nuclear staining. Because of overlapping of cytoplasmic and nuclear staining in a large number of samples, only cytoplasmic staining was evaluated. In other immunohistochemical studies, the staining in colorectal cancer, was cytoplasmic, but in pancreatic cancer, mainly nuclear (Arpalahti 2017).

Earlier, high UCHL5 expression was associated with poor survival and cancer recurrence in hepatocellular carcinoma, esophageal cell carcinoma, and epithelial ovarian cancer (Chen 2012, Fang 2013, Wang 2014). Our opposite result in gastric cancer is more in concordance with results in colorectal and pancreatic cancers achieved by a similar immunohistochemical staining method with the same antibody (Arpalahti 2017). Reasons for the differences are unknown. Study methods were different, for example Western blot in hepatocellular and ovarian cancers, and importantly, UCHL5 has high tissue-specificity in expression pattern and may play a different role in different cancer tissues.

Several studies have focused on the potential mechanism of UCHL5 in cancer. Thus far, UCHL5 and upregulation of transforming growth factor-β (TGF-β) signaling in tumors are qite clear (Wicks 2005,2006, Cutts 2011). UCHL5 and TGF-β signaling studies have showed that UCHL5-selective knockdown reduces the levels of certain TGF-β-dependent target genes, which are vital proteins in promoting tumor migration and invasion (Fang 2017). Other potential roles of UCHL5 have been studied, for example, by functional proteomic analyses aiming to screen proteins interacting with UCHL5 in cancer cells. UCHL5 promotes migration and invasion of hepatocellular carcinoma cells (Fang 2013). Thus far, the knowledge of the effects of UCHL5 in cancer is limited. Additional studies will lead to understanding of the deeper mechanism behind the effects of UCHL5 in cancer.

10.1.4 MMP-8 and TIMP-1

Gastric cancer patients with either low or high preoperative serum MMP-8 had a significantly more unfavorable prognosis. This study also showed that elevated serum TIMP-1 level serves as an independent marker of poor prognosis, as earlier demonstrated. Knowing how these two, MMP-8 and TIMP-1, interact with each other, it was interesting to calculate the molar ratio of these two molecules. Patients with low or high MMP-8/TIMP-1 molar ratios had a considerably worse prognosis. We also studied tissue MMP-8 by immunohistochemistry but found it to have no influence on gastric cancer prognosis.

Traditionally, tumor markers have used only one cut-off, separating the patients into those with good or with poor prognosis. Otherwise, some linear model would show what happens to survival when concentration of tumor marker changes. Our nonlinear serum MMP-8 results indicate a need for a physiological balance of MMP-8. A physiological level of MMP-8 is most favorable for the patient with gastric cancer, because as either excess or lack of MMP-8 favors cancer aggressiveness.

MMP-8 is an intriguing molecule with its immunoregulating and also antitumor properties. Its role in cancer has not been studied extensively. MMP-8 substrates include collagen, protease inhibitors, proteases, growth factors, cell-adhesion proteins, and cytokines, and it is expressed by a wide range of different cells, for example, neutrophils, macrophages, and plasma cells (Van Lint 2006, López-Otín

2009). Based on this knowledge that MMP-8 takes part in many biological processes, its antitumor properties do not cause surprise. As MMP-8 has an ability to modulate tumor cell adhesion and invasion and participate in inflammatory mediator processing, MMP-8-deficient mice can develop skin tumors and tongue cancer more often than do wild-type mice (Balbín 2003, Gutiérrez-Fernández 2008, Korpi 2008). In breast-cancer cells, MMP-8 expression causes a decrease in tumor growth and lung metastasis formation, providing evidence of MMP-8 antitumor function in cancer and metastasis (Soria-Valles 2014). Korpi et al. showed also that in a clinical tongue-cancer patient cohort, MMP-8 expression is significantly associated with better survival (Korpi 2008). However, the opposite findings exist, as with elevated MMP-8 expression is also linked to advanced cancer type and poor patient outcome in hepatocellular carcinoma, in colorectal cancer, and in ovarian cancer (Stadlmann 2003, Väyrynen 2012, Lempinen 2013). This phenomenon, that patients with intermediate MMP-8 survive best, has not yet been described. The role of MMP-8 in gastric cancer seems more complex than that of other MMPs.

Unexpectedly, tissue MMP-8, studied by immunohistochemistry, had no effect on patient survival. MMP-8 immunostaining was mainly cytoplasmic, and neutrophils showed MMP-8 immunopositivity, as well. Approximately half the samples were negative (57%). MMP-8 immunoexpression in malignant diseases has been studied to a lesser extent, but the cytoplasmic staining pattern tended to be similar, at least in ovarian and colorectal cancers (Stadlmann 2003, Väyrynen 2012). This result shows that the same biomarker's serum level and tissue expression do not necessarily correlate, as would have been expected. Peripheral blood MMP levels are thought to reflect local MMP concentrations in the tumor microenvironment, but differences appear in tumor tissue expression and amounts of active MMP-8 in the circulation. The active serum MMP-8 we detected may also originate from different sources related to cancer, such as from stroma, rather than from the tumor cells.

High TIMP-1 level predicted worse prognosis as expected on the basis of earlier studies (Joo 2000, Yoshikawa 2001, Wang 2006, Mroczko 2009, Kemik 2011, Grunnet 2013). TIMP-1 is one of the naturally occurring inhibitors of MMPs, and the balance between expression of MMPs and TIMPs during tumor progression is interesting. High TIMP-1 level would thus associate with tumor progression and unfavorable patient outcome, but would not cause it (Egeblad 2002). However, TIMP-1 tends to have also an independent role in cancer progression by its ability to inhibit apoptosis, to induce angiogenesis, and to stimulate cell proliferation (Yoshiji 1998, Jiang 2001,2002, Egeblad 2002, Liu 2005, Kessenbrock 2010). These independent effects may lead to cancer cell spread and cause metastasized disease.

TIMP-1 is a biomarker evaluated mostly for prognostics, but also for a predictive and diagnostic purpose in a few cases (Grunnet 2013). In gastric cancer, reports concerning serum levels of TIMP-1 show elevated TIMP-1 levels to associate with poor prognosis (Wang 2006, Mroczko 2009, Kemik 2011). One earlier study showed the prognostic value of tissue TIMP-1 as an independent factor of poor prognosis

(Mimori 1997). Apart from that, only one study, used disease-specific survival as an endpoint when calculating survival, as we did (Joo 2000). Based on these, this study strengthens knowledge of TIMP-1 as an independent prognostic biomarker in gastric cancer.

TIMP-1 binds MMPs in a 1:1 stoichiometry, and we calculated the molar ratio of these two molecules, as we already had measured the serum levels of both. We found two cut-offs for MMP-8/TIMP-1 molar ratio, and patients with a low or high molar ratio had worse prognosis than did those with an intermediate ratio, as expected after the nonlinear MMP-8 result.

10.2 Strengths and limitations of study materials and methods

The study material consists of two TMA series collected from tumor tissue samples from patients operated on during a period of 26 years (1983 to 1999 and 2000 to 2009). During these years, TNM classification, surgical techniques, and oncological treatments have, of course, developed. Based on this, one aim of this thesis project was to construct the later TMA series, collect the needed patient data, and apply it to biomarker studies. During this project, survival data and cause of death have been updated regularly. Thus one notable strength of this work is its large and well-characterized patient cohort with reliable and long clinical follow-up and survival data.

Oncological treatment of gastric cancer has changed greatly. Nowadays, guidelines recommend perioperative (pre- and postoperative) chemotherapy for patients with resectable (≥ stage IB) gastric cancer (Smyth 2016). This implies effects from chemotherapy already evident in surgical tumor-tissue samples. The behavior of antibodies in this kind of tissue may differ from that in untreated tissue. In the first TMA series, none of the patients, and in the later series only 15 patients (2.3%) received preoperative therapy; we did not exclude those.

The TMA technique, used in all four studies, is suitable to analyze large patient cohorts with a homogenous staining method, but allows analysis of only a small portion of each tumor. When considering tumor heterogeneity and the surrounding stroma, this could cause misinterpretation. With adequate sampling of at least three histologically representative spots, the TMA method allows results in concordance with those from whole-tissue samples (Kononen 1998, Kallioniemi 2001, Torhorst 2001).

10.3 Future prospects

None of the biomarkers studied in this thesis have sufficient evidence to support clinical practice as yet. More studies should examine the markers' behavior in healthy and malignant tissues as well as ways to finally apply that information to clinical practice and for the benefit of patients. Some fascinating specific questions for future prospects arose in the writing of this thesis.

PODXL would also provide promising help as a diagnostic tool. It would be interesting to investigate whether serum PODXL would also prove useful in the diagnosis of gastric cancer as it is in pancreatic cancer. One recent study showed that actually serum levels of PODXL were higher in pancreatic cancer patients than in healthy controls, and the authors suggest that increased expression of serum PODXL is more accurate for the diagnosis of pancreatic cancer than serum CA19-9 (Taniuchi 2018).

Both tissue- and blood biomarkers should ideally predict the effect of an oncological treatment such as KRAS and cetuximab in colorectal cancer, as well as HER-2 and tratszumab in breast and gastric cancers (Ross 2003, Lièvre 2008, Okines 2012). In addition to the prognostic value of TIMP-1, it has been studied also as a promising biomarker to predict its effect on chemotherapy. In breast cancer, what has been shown is that low TIMP-1 is associated with better response to antracycline-based chemotherapy (Ejlertsen 2010). In colorectal cancer, TIMP-1 plasma levels are associated with response to treatment and with survival benefit when treatment is with irinotecan and 5-fluorourasil, but not when 5-fluorourasil is combined with oxaliplatin (Sørensen 2007, Frederiksen 2011). These chemotherapy agents are treatment options also for gastric cancer; similar studies would thus be interesting and useful in gastric cancer as well.

Studies comparing expression of certain biomarkers and differences between their tissue and blood levels would be beneficial. Discrepancies can be notable, as we noticed with MMP-8. For example, in colorectal and breast cancers, no correlation exists between plasma or serum levels of TIMP-1 when compared to their tissue levels (Schrohl 2008, Sørensen 2008). These reports suggest that cancer-related factors other than only marker concentration in tissue may influence its level in plasma or serum.

The use of different combinations or even larger panels of biomarkers would enhance their reliability and value. One proposal is to combine the promising new markers with ones already in clinical practice, as a Danish study found that when combining plasma TIMP-1 and CEA protein measurements; that combination was noted as of potential aid in early detection of colorectal cancer (Nielsen 2011).

11 CONCLUSIONS

- PODXL expression in tumor tissue is an independent marker in gastric cancer of poor prognosis.

- High cytoplasmic PROX1 tumor expression is an independent marker in gastric cancer of better prognosis.

- In subgroups of stage I-II, small (< 5 cm) tumor size, and age 66 or older, cytoplasmic UCHL5 expression in tumor tissue is linked to better gastric cancer prognosis.

- Patients with either low or high preoperative serum MMP-8 have a significantly unfavorable gastric cancer prognosis.

- High preoperative serum TIMP-1 is an independent prognostic factor of worse prognosis in gastric cancer.

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